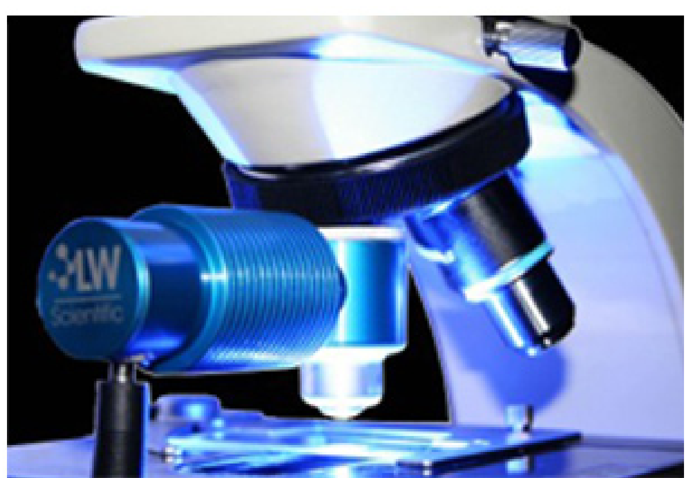

Current Research in MICROBIOLOGY

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Current Research in Microbiology

Chapter 1

Applications of Probiotic Bacteria and Dairy Foods in Health

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Abstract

The intestinal microbiota composition has a great impact on physiology and health, since commensal bacteria are crucial to maintain homeostasis and immune regulation of the gut. Consequently, disturbances of this microbiota, a process known as dysbiosis, have severe implications for the host health such as the rise of many gastrointestinal (GI) problems; including inflammatory disorders like the Inflammatory Bowel Diseases (IBD), mucositis, as well as colorectal cancer (CRC). The consumption of probiotics with beneficial effects is a promising tool to help treating such disorders. Indeed, they modulate diverse biological mechanisms involved in GI homeostasis and have been commonly used to reduce such disorders. In this chapter, we present the molecular mechanisms triggered by probiotic bacteria to modulate the gut physiology during gastrointestinal disorder and the importance of the gastrointestinal stresses tolerance as a limiting factors for probiotic application. Moreover, we focus on the emergence of functional probiotic foods, which can act as excellent vehicles, by enhancing stress tolerance and providing a protective matrix towards digestive stresses.

1. Introduction

Bacteria-host cross talk within the gut is a growing field of interest. While significant-knowledge has been achieved by studies of interactions between pathogenic bacteria and the host, much research is required for understanding the impact of commensal bacteria that reside

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within the human gastrointestinal tract (GIT) [1]. An increasing number of studies indicate that the intestinal microbiota is essential for host functions, especially immune responses that contributes to gut homeostasis [2-5]. More recently, several studies show correlations between disturbed microbiota composition (dysbiosis) and diseases which involve gastrointestinal inflammation [6-9]. Individuals presenting these inflammatory conditions are colonized by an abnormal microbiota and it has been revealed that the lack of bacteria involved in regulation of the gut immune system might be a key factor in the chronicity of mucosal inflammation [10,11]. Therefore, a novel rationale aiming at the restoration of a healthy microbiota has been glimpsed by researchers to prevent and/or help in treating gastrointestinal diseases. In this context, there has been much encouragement for the use of probiotics and functional foods as therapies for such disorders. In this chapter, we describe the most recent advances of dairy foods and probiotic strains protective effects in animal models of intestinal inflammation and in human clinical trials. Furthermore, the challenges and limitations in regard of stability and safety of these approaches are discussed.

2. Gastrointestinal Tract

2.1. Microbiota

The GIT of mammals is a complex biological system whose main function is the digestion of food. As the GIT is an environment that is very rich in nutrients, particularly the ileum and colon parts, there is a dynamic community of microorganisms, known as intestinal microbiota, which plays a role in the intestinal physiology and immune regulation [12-14].

The community of bacteria found in the GIT contains both indigenous and transient members. The first ones are well adapted to the intestinal environment and thus colonize the lumen. In turn, the transient microorganisms are not able to survive more than a few days. Some transient species are frequently ingested in substantial amounts as they are present in fermented dairy foods such as yogurts, cheeses and fermented milk. However, transiting bacteria also include several of the enteric food-borne pathogens [15,16]. The survival of allochthonous bacteria in the GIT depends on several factors including the ability to tolerate gastric acid, bile salts and pancreatic juice [17].

Many academic and industrial consortiums, such as the MetaHIT (Metagenomics of the Human Intestinal Tract), have attempted to characterize the microbiota associated with the human GIT through genomic sequencing, thus giving a more detailed description of the human intestinal microbiota composition and of its function [18,19]. It is estimated that the intestinal microbiota comprises 500 to 1,000 species of bacteria, exceeding 10 times or more the total number of host cells [7,20,21]. Nowadays, it is known that most species found in mammalian GIT can be classified into four phyla: Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria [21-23]. These phyla keep symbiotic relationships with the host, making

fundamental contributions to the host metabolism while occupying a protected environment rich in nutrients [24,25]. In this context, the intestinal microbiota plays major roles such as nutritional functions, prevention of pathogen colonization, trophic functions on the proliferation and differentiation of the intestinal epithelium, and development and modulation of the host immune system [21,26-9].

Although the microbiome composition varies greatly among individuals, its composition is relatively simple during the first years of life. It has been reported that *Escherichia coli* and streptococci are the most common organisms isolated from the upper GIT shortly after birth [21,30]. These species are responsible for creating a favorable environment, promoting in turn the colonization by anaerobic bacteria, among which *Bifidobacterium* and Bacteroidetes are most prevalent. The *Bifidobacterium* genus plays an important role in the intestines, as these bacteria can down-regulate the expression of key proinflammatory mediators in the gut and inhibit pathogens. Bacteroidetes species have great capability to digest complex sugars, and thus maintain stable symbiotic relationship with the host and central position in the gut microbiome [31-34]. Afterwards, in the course of life, there can be an increase of Firmicutes, especially of the Lactobacillales and Clostridiales Orders. Among these, several members, including remarkably *Faecalibacterium prausnitzii*, are Short Chain Fatty Acid-producers, SCFAs playing an important role on the maturation of regulatory T cells [35]. Although microbiota is stable in older persons, it can be altered in short term duration by dietary intervention [36]. Dietary intake shifts, mainly of dairy foods containing *Lactoccocus* sp or *Propionibacterium* sp and non digestible carbohydrates, may change the composition of the gut microbiota by increasing the number of *Bifidobacterium* sp and *F. prausnitzii,* although these bacteria do not respond in the same way in all human subjects [27,37,38].

In adulthood, the diversity and abundance of bacterial populations vary along the different parts of GIT [13,21,39,40]. In the stomach and duodenum, a small number of microorganisms can be found, while up to 10^3 bacterial cells are present per gram of duodenal content. These bacteria are adhered to the mucosal surface or in transit through the GIT [26,41,42]. Streptococci and lactobacilli are among the most common groups of bacteria found in this part of the intestine [39,43-45]. The bacterial population increases gradually along the jejunum and the ileum, reaching numbers around 10^4-10^7 per gram of small intestinal content. However, it is in the lower GIT (colon) that the highest density of bacteria population is encountered, reaching a number of 10^{11}-10^{12} per gram, making this area one of the most complex microbial ecosystems known to date on Earth [13,45].

2.2. Immune system regulation

Commensal species from the intestinal microbiota are not ignored by the immune system but on the contrary need to be recognized by mammalian cells to deliver tolerogenic sig-

nals and promote intestinal immune homeostasis with an impact on the immune system of the body. Actually, commensal bacteria and their host have co-evolved diverse biological mechanisms making this cross talk possible [46]. For example, pattern recognition receptors (PRRs), especially Toll-like receptors (TLRs), expressed by Intestinal Epithelial Cells (IECs), are able to recognize microbe-associated molecular patterns (MAMP) of the commensal microbiota. In fact, the expression of PRR and their interaction with the microbiota is very important for the healthy development of the host immune system [47-49].

These MAMP are microbial components, such as lipoproteins, nucleic acids (RNA and unmethylated CpG dinucleotides), lipopotheic acids, lipopolysaccharide (LPS), surface proteins such as flagellin and peptidoglycan [7,50-52]. The recognition of a MAMP transduces signals that subsequently activates innate immune responses [50,52].

Species from Lactobacillales order and the Actinobacteria phylum (*Bifidobacterium* sp. and *Propionibacterium* sp.) [30,53] are capable of stimulating luminal secretion of antimicrobial peptides by Paneth cells, mucins by goblet cells and fortifying tight junctions of IECs [54, 55]. Furthermore, commensals are reported to induce signals of immunological tolerance, such as secretion of the TGF-β cytokine that inhibits the NF-κB signaling pathway inside epithelial cells. It has been shown that activation of TLRs by commensal bacteria also promotes the development of CD103 dendritic cells, which are responsible for driving the activation of Treg cells [56-58]. It is known that Treg cells suppress effector T cell responses mainly through the production of IL-10 and TGF-β. The stimulation of these cytokines prevents the recruitment of granulocytes, suppressing the activation of macrophages, neutrophils and endothelial cells. In addition, Treg cells expressing TGF-β and IL-10 drive B cells to undergo antibody class switching to produce IgA antibody, the major humoral defense of mucosal surfaces. Secretory IgA (sIgA) contributes to mucosal homeostasis through a process known as immune exclusion. sIgA is able to bind to opportunistic pathogens avoiding its dissemination throughout the body [59-61].

Although commensal microorganisms show beneficial effects on the host, some microbes of the GIT might present potential risk if case of outgrowth. In this context, potentially pathogenic species, known as pathobionts, composed mainly of Proteobacteria members, such as *Escherichia coli*, and species from the phylum Firmicutes, as *Clostridium difficile* and *Enterococcus faecalis* can elicit a pro-inflammatory immune response after binding to TLR [47, 53,62].

When pathobionts translocate to intestinal epithelium, the host immunity is activated and is usually enough to eliminate the intruder. Nonetheless, the overproduction of pro-inflammatory cytokines, which may occur during dysbiosis, represents a risk once inflammation may also be problematic causing cell disruption and infection to the host. Therefore, to reach intes-

tinal homeostasis, the gut immune system must be able to recognize and eliminate specifically these pathobionts from the GIT [54,63]. Intestinal barrier dysfunction generates an imbalance between immune responses observed for protective and harmful intestinal bacteria and thus contributes to the onset of several inflammatory conditions of the GIT [7,64].

2.3. Inflammatory disorders

The GIT is permanently challenged by antigens from the intestinal microbiota. Under normal conditions, the intestinal mucosa maintains tolerance to commensals, mainly through the action of Treg cells. When the dynamic balance between Treg and activated effector cells is broken, the homeostasis is compromised and this may lead to the development of mucosal inflammation [2]. Besides dysbiosis, multiple factors can influence the proper functioning of the GIT immune system, including individual genetic background, diet, use of drugs and environmental stress. The intersection of these factors generates an exaggerated pro-inflammatory reaction against commensal antigens leading to Inflammatory Bowel Diseases (IBD), a group of chronic inflammatory conditions of the GIT, which primarily includes ulcerative colitis (UC), and Crohn's disease (CD) [21,30]. Clinical symptoms of both diseases are similarly found in patients, such as abdominal pain, diarrhea, rectal bleeding and weight loss [65,66]. Relapse symptoms can last days, weeks, or even months [67]. CD is characteristically discontinuous, with inflamed areas that can be found in all the layers of the intestinal wall, while UC is characterized as a continuous and superficial inflammation limited to the colon [68,69]. The incidence of these diseases varies widely across countries, however, in recent years, it has increased considerably worldwide, being considered a global public health problem. This increase has been associated with the modern lifestyle that includes the ingestion of processed foods usually high in fats and sugar and low in fiber [70-72].

Caesarean delivery and the inappropriate use of antibiotics, especially during childhood, when the microbiota has not yet been established, are factors that can contribute to the development of intestinal inflammation [30,72–74]. Both CD and UC have different immunological aspects when it comes to innate and adaptive immunity [75,76]. Pro-inflammatory cytokines are over expressed in IBD patients; however, the predominant set of cytokines observed in CD patients is the one secreted by Th1 and Th17 cells (IL-12, IL-23, IL-27, IFN-γ) whereas in UC patients a Th2 immune response, characterized by the production of IL-4 and IL-13, appears to be predominant [3,4,74].

Chronic inflammation also plays a role in the pathogenesis in several cancers and it has been shown that there is a direct link between IBD and colorectal cancer (CRC) [77]. Individuals suffering from long-term ulcerative colitis or Crohn's disease have increased risk of developing CRC [78].

Reactive Oxygen Species (ROS), stimulated by proinflammatory response in the intes-

tinal mucosa, play an important role in the development of CRC, as their excessive levels can result in oxidative stress and significant damage to cell structures and macromolecular constituents, such as DNA, RNA, proteins and lipids [79,80]. Large amounts of hydrogen peroxide (H_2O_2) are produced and excreted by human tumor cells, and might participate in tumor invasion and proliferationas well [78,81]. Furthermore, current studies are investigating the role of effector immune responses against intestinal microbiota in modulating the gut microbiota into a carcinogenic profile composition. In fact, a correlation between diet-driven sulfidogenic bacteria and CRC in African Americans has been demonstrated [82,83].

Other factors such as the use of some medicines can also contribute to the breakdown of this immunological tolerance against commensals commonly observed under normal conditions. It has been described that chemotherapeutic agents, as 5-Fluoracil (5-FU), doxorubicin and irinotecan (CPT-11), widely used in the treatment of advanced solid tumors, may also lead to the development of another inflammatory condition of the GIT, known as mucositis. This painful inflammation of the mucosa can affect all portions of the human GIT and has great medical importance as it arises as an adverse effect of chemotherapy [84,85]. These medicines are effective in cancer treatment because they inhibit cell proliferation. 5-FU, for example, causes cytotoxic effect by inhibiting DNA replication in cells with a high mitotic index such as malignant cells. Moreover, this drug can also be incorporated into RNA molecules interfering with their processing and function. However, as an adverse consequence, the drug also shows an effect in normal cells that presents a higher turnover rate, such as GIT enterocytes [86]. Gastrointestinal mucositis is being regarded as a major risk, occurring in 80% of patients receiving 5-FU [87,88].

Patients with mucositis develop symptoms like odynophagia (pain in swallowing), vomiting, abdominal pain and diarrhea, which make eating difficult. Therefore, weight loss and malnutrition are also reported and quality of patient`s life is Gastrointestinal mucositis is characterized by morphological alterations in the mucosal architecture, as villous atrophy, increased cryptsapoptosis, that expose the mucosa to intestinal pathogens, which are able to translocate across intestinal epithelial cells leading to inflammatory responses [89].

The pathophysiology process of mucositis is very complex and involves the release of endogenous damage-associated molecular pattern (DAMP) molecules and activation ofthe NF-kB pathway, which induces in turn the expression of several genes, including pro-apoptotic enzymes such as caspases, tumor necrosis factor-alpha (TNF-α), IL1-β and IL-6 cytokines, and chemokines involved in the recruitment of neutrophils and eosinophils. Commensal bacteria might have a very important role in promoting many clinical aspects involved in the pathogenesis of mucositis [90-94], as it was demonstrated that germ-free mice are more resistant to mucositis induction [85,95]. Moreover, opportunistic species belonging to the gut microbiota, such as *Entercocccus faecalis, Escherichia* sp and *Clostridium* sp can alter intestinal

permeability during anticancer treatments and promote disruption of the epithelial layer. The translocation of commensals across IEC exacerbates inflammatory responses and amplifies the damage to the intestinal mucosa [92,95].

As alterations of the intestinal microbiota have been implicated in all of these pathologies, the scientific community has been investigating the use of probiotics in order to restore the original gut microbiota, which is responsible for regulating the mucosal immune system.

3. Probiotics

The administration of probiotics for treating gastrointestinal inflammatory disorders have been proposed by many research groups, as they are able to occupy niches that compete with pathogens in the GIT [96-101]. Probiotics are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" [102]. These microorganisms must be safe and present beneficial effects during their transit through the gut. Hence, their ability to resist the stomach and intestine environments are crucial alongwith the capacity of adhesion to intestinal cells, inhibition of pathogens and immunomodulatory effects [103]. Currently, several species of probiotic bacteria are used to prevent or treat a diversity of diseases, including gastrointestinal inflammatory disorders (**Table 1**). Lactobacilli and bifidobacteria, for a long time, were at the front of the stage in the field of this probiotic action. However, outsider bacterial species such as *Lactococcus lactis*, *Streptococcus thermophilus*, *Escherichia coli* and *Propionibacterium freudenreichii* recently revealed promising potential for the treatment of intestinal inflammation as well [104-107]. This is summarized in section 4.

Table 1: Immunomodulatory effects of probiotics in experimental animal model

Probiotic species	Model system	Probiotic effect(s)	Mechanisms involved	Reference(s)
Escherichia coli M17	DSS	Attenuates colitis	Inhibits NF-κB, decreases colonic IL- 12, IL-6, IL1-β and IFN-γ	(FITZPATRICK et al., 2008) (108)
Lactobacillus casei	TLR4 KO and DSS	Attenuates colitis	Reduces proinflammatory cytokines secretion and neutrophil recruitment	(CHUNG et al., 2008) (109)
Faecalibacterium-prausnitzii	TNBS	Attenuates colitis	Increases colonic IL10 and decreases colonic IL12. Tends to correct the dysbiosis associated with TNBS colitis	(SOKOL et al., 2008) (110)
Mix of four *lactobacillus* or four *Bifidobacterium species*	DSS	Attenuates colitis	Reduces colonic proinflammatory cytokines	(NANDA KUMAR et al., 2008) (111)
VSL#3	TNBS	Attenuates colitis	Increases production of IL-10 and Tregs	(DI GIACINTO et al., 2005) (112)

Lactobacillussali-varius Ls33	TNBS	Attenuates colitis	Increases IL-10 production and Tregs	(MACHO FERNANDEZ et al., 2011) (113)
Lactobacillus plantarum DSM 15313*, Lactobacillus fermentum* 35D	DSS	Attenuates colitis	Reduces bacterial translocation	(OSMAN et al., 2008) (114)
Bacteroides fragilis	TNBS	Attenuates colitis	Increases production of IL-10 and Tregs	(ROUND and MAZMANIAN, 2010) *(115)*
Lactobacillus salivarius 433118*, Bifidobacterium infantis*	IL-10 KO	Attenuates colitis	Reduces inflammatory cytokines	(MCCARTHY et al., 2003) (116)
Lactobacillus casei Shirota	DSS	Attenuates colitis	Reduces IL-6 production by lamina propria mononuclear cells	(MATSUMOTO et al., 2005)(117)
Enterococcus faecium CRL 183	1,2 dimethylhydrazine (DMH)	Reduces ACF and adenocarcinomas incidence	Improved the immune response by increasing IL-4, IFN-γ, and TNF-α production	(SIVIEIRI et al., 2008)(118)
Saccharomyces boulardii	C57BL/6J Min/+ (Apc-Min) mice (7 wk old).	Reduces number and diameter of the tumors, the score for low-grade dysplasia, numbers of polyps, and cell proliferation	Inactivation of the EGFR-Mek-Erk pathway signaling. Increase apoptosis	(CHEN et al., 2009)(119)
Lactobacillus acidophilus NCFM	CT-26 cells	Reduces tumor size and the extraintestinal metastatic tissue	Increase apoptosis through increase caspase-9 and caspase-3 and reduces Bcl-2 expression	(CHEN et al., 2012) (88)
Lactobacillus plantarum AdF10 and *Lactobacillus rhamnosus* GG	1,2 dimethylhydrazine (DMH)	Reduces tumor incidence, multiplicity, and size	Reduces COX-2 protein expression	(WALIA et al., 2015) (120)
Lactobacillus salivarius Ren	1,2 dimethylhydrazine (DMH)	Reduces tumor incidence	Reduces Intestinal population of Ruminococcussp and Clostridiales bacteria ↑ Intestinal population of Prevotellasp	(ZHANG et al., 2015) (121)
Dead nanosized *Lactobacillus plantarum*	Azoxymethane/ Dextran Sulfate Sodium-Induced	Reduces tumor incidence; areas of dysplasia, adenocarcinoma, and structural disruption	Reduces Over expression of proinflammatory cytokines and inflammatory genes Increase Apoptosis and cell cycle arrest	(LEE et al., 2015) (122)
Lactobacillus rhamnosus and *Lactobacillus acidophilus*	1,2 dimethylhydrazine (DMH)	Reduces tumor incidence, burden and multiplicity; lipid peroxidation	Reduces GSH, SOD, and GPx activity	(VERMA and SHUKLA 2014) (123)

Lactobacillus casei BL23	1,2 dimethylhydrazine (DMH)	Reduces colorectal cancer	Regulates Treg and Th17 T-cell populations	(LENOIR et al., 2016) (124)
Pediococcus pentosaceus GS4	Azoxymethane	Attenuates colon cancer	Triggered apoptosis in colonocytes	(DUBEY et al., 2016) (125)

3.1. Bacteria for the treatment of gastrointestinal disorders

Many strains of bacteria are known to exert anti-inflammatory effects through the modulation of factors that are involved in maintaining intestinal homeostasis in humans and other animals [97,101]. In this context, bacterial effectors of distinct nature have been implicated in probiotic effects. These include metabolites, peptidoglycan, surface proteins, lipoproteins and lipoteichoic acids, lipopolysaccharides, flagelin and CpG motifs in DNA. Some of these molecules, such as anti-microbial peptides and prebiotic metabolites may interact directly with other species of bacteria that colonize the gut, modulating their growth. Others bacterial factors, called MAMP, bind to PRRs of eukaryotic cells and stimulate different patterns of gene expression in the host involved in innate immunity activation and differentiation of antigen-specific immunity [53,126]. The mechanism of action of these bacteria can be classified in three main categories: alteration of gut microbiome composition, stimulation of epithelial barrier function; and induction of the immune responses [127].

Recent advances in genomic sequencing technologies have provided the scientific community with tools to explore the human microbiome and how different treatments affect its global composition and function. Several studies have shown that probiotics can increase or decrease the abundance and diversity in gut microbial species composition. The secretion of antimicrobial compounds acts by directly inhibiting the growth of pathogens. In addition, probiotic strains may also reduce the impact of pathogens through a mechanism known as competitive exclusion, in which they occupy binding sites at the mucosal surface [97,128,129].

Epithelial barrier function enhancement is a well-established mechanism of probiotic bacteria in the protection of the host against invasive harmful bacteria. Numerous studies have shown that probiotics have the potential to modulate many of the processes involved in mucosal barrier formation and are able to upregulate expression of defensins, mucins or proteins associated with tight junctions such as claudins and occludins [130-132]. This effect is therefore considered as one of most important for the prevention and treatment of IBD and mucositis, as it might avoid translocation of opportunistic pathogens to systemic circulation [84,101].

Probiotics can affect the host heath by modulating inflammatory signaling pathways. Several probiotics are reported to inhibit the NF-kB activation and thus to influence downstream cytokine secretion [133]. Recent studies demonstrated that the anti-inflammatory effects of some bacteria involve inhibition of IkB degradation by targeting the different steps involved

in this process which are phosphorylation, ubiquitination or proteasome degradation[134]. Some Lactobacilli have shown inhibitory activity of TNF-alpha induced secretion of IL-8 [135]. Other well established immunological mechanism of probiotics is the stimulation of immunological tolerance to GIT microbiota through the increase in IL-10 secretion. For instance, Santos and collaborators (2014) showed that the probiotic effect of *L. delbrueckii* strain CNRZ327 was related to an expansion of Treg cells and an increase of total IgA in Dextran sulfate sodium (DSS)-induced colitis in mice. Recently, it was reported that a *Lactococcus-lactiss* sp. lactis NCDO2118 strain prevented DSS-induced colitis in mice and the protective effect was related to increased IL-10 levels in the colon and to the induction of Treg cells in the mesenteric lymph nodes [99].

Most studies focused on the beneficial effects autochthonous Lactobacilli (**Table 1**). However, recent studies have demonstrated that some allochthonous strains have anti-inflammatory properties. Ballal and colleagues (2015) found that *L. lactis* I-1631 prevents colitis in T-bet−/− Rag2−/− mice. Two additional studies have shown that, among the *L. lactis* species, NCDO2118 subsp. *lactis* or FC subsp. *cremoris* are anti-inflammatory when inoculated in inflamed mice receiving the chemical agent DSS [99,136]. Moreover, *L. lactis* NZ9000 by itself was able to prevent histological damage and reduce neutrophil and eosinophil infiltration in mice injected with 5-FU. Another allochthonous species with anti-inflammatory effects in IBD models is *Propionibacterium freudenreichii,* used extensively as a ripening starter of Emmental cheese [104,137,138].

3.2. Challenges and limitations to select probiotic bacteria

For probiotic bacteria selection, the robustness of a bacterium against different abiotic and biotic stresses is crucial, and may constitute a limiting factor for its application as probiotic. Firstly, to prepare probiotic ingredients, a plethora of stresses are applied, thus the bacterial tolerance is a prerequisite for reaching a high survival rate in the product. In the traditional cheese products, the manipulation of bacterial population could be limited by other factors. However, for the probiotic powders, great efforts were made to maintain a high viable bacterial population during freeze-drying or spray drying, such as usage of encapsulation methods. Gastrointestinal stresses also constitute the main bottleneck of probiotic efficacy.

A probiotic microorganism must be able to tolerate digestive stresses and to adhere to intestinal epithelium, for a long persistence in the host and for an enhanced beneficial effect. Gastric acid and bile salts are defense mechanisms encountered during intestinal transit whereas pancreatic secretions can also exert some antimicrobial activity via the digestive enzymes. The existing microbiota may also interfere with the probiotic effect by competition for adhesion or nutrients. The investigation of molecular basis of the adaptive response to stresses and identification of the pivotal genes involved provided pertinent tools for probiotic screening.

3.2.1. Acid stress

The probiotic resistance to acid stress is a desired characteristic of selected strains, as low pH is widely encountered both during technological processing and during gastric digestion. The bacterial adaptive responses to acid challenge have been investigated and some of the molecular mechanisms involved were elucidated, such as induction of proton ATP-dependent pumps F_1F_0-ATPase. The function of this transmembrane protein complexis the extrusion of protons from the cell cytoplasm, resulting in a Proton Motive Force (PMF), and avoiding acid-stress induced drop in intracellular pH [139]. Mutations leading to a reduction of membrane-bound ATPase activity were observed in some strains of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus helveticus*, where they cause growth inhibition under acid conditions [140,141]. Gram-positive bacteria, such as *Lactococcus lactis* [142] and *Lactobacillus brevis* [143], possess a second mechanism for an adaptive response to acid stress, involving the enzyme glutamate decarboxylase (GAD). The GAD system imports glutamate into the cell prior to its decarboxylation, which consumes protons, participating in intracellular pH homeostasis, followed by the efflux of the resulting γ-aminobutyrate (GABA), thanks to a GAD/GABA antiporter. Another mechanism involved in pH homeostasis is the proton-consuming malolactic fermentation (MLF). This metabolic pathway leads to the conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid. The latter is excreted via a lactate-malate antiporter, resulting in intracellular alkanization. Such mechanism was observed within several bacteria like *Lactobacillus sakei* [144], *Lactobacillus plantarum* [145], and *Lactococcus lactis* [146]. Finally, other acid-adaptive mechanisms induced in lactic acid bacteria includethe citrate-lactate antiporter (CitP), the arginine deiminase (ADI) system and some heat shock [147-149].

3.2.2. Bile salts

Conjugated bile salts are synthesized by the liver with the amino acids glycine or taurine, those amphipathic molecules act like biological detergents with strong antimicrobial activity cause can emulsify biological membrane lipids [150,151]. These compounds may enter into the bacterial cytoplasm by flip-flop mechanism and cause oxidative stress which leads to DNA damage [152-154]. In fact, there are different remarkable mechanisms leading to bile salts tolerance; those molecular actors can also provide bacteria a cross protection towards other stress types. Some probiotic bacteria hold the ability to hydrolyze bile salts by bile salt hydrolases (BSHs) which enhances their survival in the digestive tract [155]. Alternative mechanisms exist such as bile-efflux systems, which are multidrug transporters that mediate the active extrusion of bile salts from the bacterial cytoplasm [156]. Regarding *Lactobacillus acidophilus* particularly, an eight-gene operon encoding for, a two-component regulatory system, a transporter belonging to the major facilitator super family, an oxido reductase, and four hypothetical proteins, has been implicated in bile salts removal [157].

3.2.3. Heat stress

Heat stress is another type of ordeal that is commonly suffered during technological processes, either during food fermentation (cheese cooking-step) or during drying, which may impose high temperatures (>60°C) or low temperatures, depending on the chosen technology. The response to heat stress involves a set of proteins called Heat Shock Proteins (HSP), which include chaperones and proteases. They are essential for overcoming protein denaturation, maintaining cell homeostasis in response to variations of temperature, which can affect membrane fluidity and compromise cellular integrity and basic cell processes [158,159]. Among those crucial proteins, DnaK and GroEL, are two HPSs that have a critical role in cellular processes by maintaining DNA replication process, preventing mutagenesis and preventing protein denaturation [158,160]. Otherwise, low temperatures are frequently used to prevent spoilage during frozen and freeze-dried storing process. Such a cold stress leads to induction of specific proteins called cold shock proteins (CSPs). Their role consists in maintaining transcription and translation processes under cold stress adaptation [159].

3.3. Protective matrix and vectorization

Although the adaptive response to various stresses is a quite important feature to screen tolerant or sensitive probiotic strains, vehicle matrix can confer a protection for an efficient delivery of probiotic bacteria to the GIT. Probiotics are commonly consumed under the form of dried powder, in capsules or tablets. Recently, various studies focused on “2-in-1”starter bacteria: microorganisms widely used in food fermentation and which exert beneficial effects. There is a huge variety of fermentative microorganisms known for their probiotic properties like *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. lactis,* and other strains used for specific fermented foods [161]. The growth in such stressful medium as dairy fermented foods selects bacteria with a high robustness to GIT stresses there by promoting long-term survival during storage in industrial process.

3.3.1. Encapsulation by biopolymers

Encapsulation is a standard process used to produce protected dried probiotic ingredients. Encapsulation may confer a protection against industrial stresses during the drying process, and allow a controlled release in the GIT [162,163]. Depending on the type of drying technology (capsule spray-drying, emulsification, extrusion, co-extrusion, or spray-coating), different particles sizes may be obtained and interfere in the encapsulation yield. Moreover, semi-permeable and biocompatible matrices including food-grade biopolymers like alginate, pectin and cellulose acetate phthalate are used for preventing oxidative reaction, masking flavor and odor changes. The encapsulation essentially provides a protection for bacteria and a specific addressing of active probiotic compounds to specifics sites [164,165]. Among polymers used for encapsulation; alginate, a polysaccharide composed of β-D-mannuronic and

αL-guluronic acids, is widely used, because of its simplicity, biocompatibility, low cost, and non-toxicity. Recently, the encapsulation by alginate was shown to confer enhanced viability upon storage and simulated gastrointestinal digestion for *Lactococcus lactis* subsp. *cremoris* LM0230, *Lactobacillus casei* NCDC 298, *Bifidobacterium longum* and other probiotics [166-168].

3.3.2. Encapsulation by milk proteins

The utilization of milk proteins for probiotic encapsulation is a high quality choice due to their biocompatibility, structural and physico-chemical properties [169]. Milk proteins are categorized in two types: caseins and whey proteins. Caseins are a complex aggregate of phosphoproteins and are extremely heat-stable proteins, present in colloidal form known as caseins micelles in fresh milk [170]. Whey proteins are a group of globular proteins, α-lactalbumin, ß-lactooglobulin, immunoglobulins, and serum albumin and also various other minor proteins [171]. Milk proteins clotting followed by spray-drying appears as new innovative methodology to encapsulate probiotic bacteria to enhance survival in GIT [172]. Heidebach *et al.* in 2009 demonstrated new methodologies based on a transglutaminase-catalyzed gelation of casein suspensions and spray drying to encapsulate *Lactobacillus paracei s*sp. *paracasei* F19 and *Bifidobacterium lactis* Bb12. It was shown to enhance robustness instressing conditions [173,174].

3.3.3. Dairy Fermented foods

The emergence of functional foods concept such as fermented products is a promising research area. Indeed, the dairy fermented foods constitute an important part of our daily diet [175], as well as our main microbial daily intake. The dairy product matrix may increase tolerance of bacteria towards digestive stresses and adhesion to cells, depending on its biochemical composition, its physical microstructure and the existing microbial ecosystem, which affects directly the viable bacterial amount reaching the gut. Beyond the protection effect of the vehicle matrix, fermentation allows improvement of food nutritional value through the microbial release of a high amount of essential nutrient for consumers [176], including vitamins.

Probiotic bacteria convert different molecules, producing valuable nutrients like conjugated fatty acid, B-galactosidase enzyme, beneficial dairy peptide, which can enhance their probiotic functionality [24,25]. Indeed, *L. casei* BL23 incubated in milk reduced significantly the symptoms of a dextran sulfate sodium (DSS) - induced colitis in a murine model, compared to the same strain provided in phosphate buffered saline [178,179]. Yogurt enhances the therapeutic value of some probiotic bacteria, however the low pH of yogurt decreases viable population [177,180,181]. To contend this problem, a combination of encapsulated probiotic bacteria was used to increase survival in yogurt. For example, *L. paracasei* subsp. *paracasei* E6 were encapsulated using whey proteins and gum arabic, before being added to the yogurt

matrix after fermentation. This bacterium exhibited greater viability, compared to cells without encapsulation, upon exposure of the probiotic yogurt to simulated gastric juice [182,183].

Cheese matrix favors probiotic beneficial effects by providing a favorable environment with relatively high fat content, enhancing probiotic survival in transit through the GIT, especially towards lethal conditions of the stomach [184]. Özer and colleagues showed increased viability of *Bifidobacterium bifidum* BB-12 and of *Lactobacillus acidophilus* LA-5, when these bacteria were microencapsulated in white-brined cheese, compared to same strains without encapsulation protection [185]. Other strains have been used to produce experimental probiotic cheese, including *Lactobacillus casei* and *Lactobacillus acidophilus* in Crescenza cheese. They exhibited improved viability, during the refrigerated storage, after a cheese manufacture using High-pressure homogenization (HPH) as an alternative to traditional thermal treatment [186]. The potential probiotic *P. freudenreichii*, alone or in combination with *Lactobacillus delbrueckii*, was investigated with respect to the prevention of UC. The experimental fermented cheeses exhibited promising anti-inflammatory properties in mice with colitis [187,188]. Moreover, inclusion within a cheese enhanced *P. freudenreichii* tolerance towards digestive stresses and thus its probiotic properties [105].

4. *Propionibacterium Freudenreichii* for Treating Gastrointestinal Disorders

During the last two decades, an outsider, that had until then been ignored, was considered for probiotic applications. The propionibacterium *P. freudenreichii,* until then used almost exclusively to confer aroma to pressed cheeses, joined the main probiotic actors on the stage of probiotic research and development. *P. freudenreichii* indeed recently revealed unexpected immunomodulatory effects. This evidenced for the first time a “two-in-one” property of an ill-known ripening starter, with both technological and probiotic abilities.

4.1. General aspects of *P. freudenreichii*

4.1.1. Taxonomy

P. freudenreichii is a dairy propionibacterium, which belongs to Actinobacteria, characterized as gram-positivewith a high G+C content, non- sporing, anaerobic to aerotolerant, nonmotile pleomorphic rods [189,190]. Actinobacteria comprise bacterial species with a mycelium-like aspect, found in various environments, including animal hosts and soil [189,190]. The genus *Propionibacterium* comprises both cutaneous species, which may act as opportunistic pathogens, and dairy species, which have no reported adverse effects up to date [191]. The typical dairy species isolated from milk are: *P. freudenreichii, P. acidipropionici, P. jensenii* and *P. thoenii;* they are clearly distinct from cutaneous species. Dairy propionibacteria were firstly described by E. von Freudenreich and S. Orla-Jensen at the end of 19th century, since their presence in Emmental cheese was associated with propionic fermentation [192]. Dairy

propionibacteria, specifically *P. freudenreichii*, possess a long history of safe use in food, particularly by Swiss-type cheese. *P. freudenreichii* received the “Generally Recognized As Safe” (GRAS) status [193]. The European food safety authority has granted “Qualified presumption of safety” (QPS) status to two species: *P. freudenreichii* and *P. acidipropionici* [194]. The sequencing of *P. freudenreichii* genome revealed the genetic basis of the great adaptation ability to various environments [195]. They moreover display a peculiar fermentative metabolism, which relies on propionic fermentation, may use various carbon and energy sources, and release in the extracellular medium various beneficial metabolites [190,195].

4.2. *P. freudenreichii* technological applications

4.2.1. Swiss-type cheese manufacturing

The major use of *P. freudenreichii* strains is as ripening culture in Swiss-type cheeses manufacturing. They play an important role in characteristic flavor of cheeses such as Emmental cheese [189]. *P. freudenreichii* produces several flavor compounds via different substrates catabolism. lactate and aspartate fermentations generate short fatty acids accumulation, mainly propionic and acetic acids, and to a lesser extent valeric and isovaleric acids. These short fatty acids are considered as principal flavor compounds in Emmental. *P. freudenreichii* also possesses a strain-dependent lipolysis activity, which produces free fatty acids that are important molecules for cheese flavor. The amino acids catabolism by *P. freudenreichii* produces two branched-chain flavor compounds: 2-methylbutanoic acid and isovaleric acid [190]. In Emmental cheeses, *P. freudenreichii* reaches a high population density, with counts depending in ripening period. The *P. freudenreichii* robustness permit a high tolerance to different stresses during cheese manufacturing process, such as high and low temperature, acidification, osmotic stress induced by NaCl [190,196]. In addition, *P. freudenreichii* can also be found in low amount in various cheeses, in addition to Emmental cheese [189].

4.2.2. Anti-microbial & Nutraceutical molecules production

P. freudenreichii is a well-known vitamin B_{12} producer, actually, the only B_{12} producingbacteria with the GRAS status [190,197]. Vitamin B_{12} is an essential vitamin, required for maintaining healthy nerve cells, DNA synthesis and energy, and for other important functions. Vitamin B_{12} is synthesized industrially by chemical synthesis, which is too difficult and expensive. Many efforts were made to enhance the productivity of vitamin B_{12}, by using genetic engineering and by optimizing fermentation conditions. In addition, *Propionibacterium* spp strains, have preservatives properties and are widely employed to extend foods shelf-life by inhibiting undesirable microorganisms growth. A commercial product is available under MicrogardTM name, which is composed of skim milk fermented by *P. freudenreichii* subsp *shermanii* [190,192,197]. The short chain fatty acids propionate and acetate, as well as other organic acids such as succinate, are the main anti-microbial molecules produced by dairy pro-

pionibacteria. However, *P. acidipropionici* species were shown to be the best producer of propionic acid, through glycerol fermentation without acetic acid production [197]. Different bacteriocins that are produced by both dairy and cutaneous propionibacteria, have been reported and characterized [190,192,197]. However, further studies are required to assess their possible use as food biopreservatives or bacteriocin producer probiotics to inhibit intestinal pathogens, as dairy propionibacteria bacteriocins are not still recognized as GRAS by the FDA.

4.3. Probiotic application

Recent data suggest the probiotic potential application of dairy propionibacteria, mainly *P. freudenreichii*, for human and animal, as this species presents all characteristics for probiotic application [192]. Indeed, it shows a high tolerance to digestive stresses, which is one of the main factors limiting the use of microorganisms as live probiotic agent [192]. Propionibacteria species have a slow growth rate, so their adherence to intestinal epithelium is crucial for their persistence in the gut and for exerting their beneficial effects [190,192]. Some studies demonstrated the dairy propionibacteria ability to adhere to intestinal cells, however all those studies are *in vitro* experiments, and the adhesion presented a lot of variations according to adhesion model used, species types, and vehicle or growing medium [192]. *In vivo* studies, in humans and mammalians, suggest that this adhesion ability allows only a transient colonization, since fecal propionibacteria population in human volunteer's decreases after ceasing the ingestion of propionibacteria [198,199]. *P. freudenreichii* produces several beneficial metabolites, allows specific changes, as microbiota and intestinal immunity modulations [192]. Some strains of dairy propionibacteria are already used in probiotic preparations, alone or in combination with lactic acid bacteria and/or bifidobacteria [192]. Recently, the spray-drying was shown as a better alternative method to dry probiotic bacteria, since energy costs are lower and the process is sustainable [200,201]. *P. freudenreichii* was shown to tolerate stresses undergone during different technological stresses, which will lead to the development of several fermented ingredients to exert probiotic potential of dairy propionibacteria for improving animal and human health.

4.3.1. Molecular mechanisms of *P. freudenreichii* beneficial effects

Regarding *P. freudenreichii*, animal studies and clinical trials indicate its ability to modulate gut immunity and microbiota, specifically in the context of UC. *P. freudenreichii* was shown to prevent trinitrobenzene sulfonic acid (TNBS) induced colitis in conventional mice, alone or associated with other probiotic bacteria [202,203]. Immunomodulation by *P. freudenreichii* was further evidenced in pigs, by decreasing plasma haptoglobulin and proinflammatory cytokines (IL-8 and TNF-α) in gut mucosa, after lipopolysaccharides (LPS) stimulation *ex vivo* [198]. Recently, a probiotic mixture containing both *Lactobacillus rhamnosus* and *P. freudenreichii* was tested in humanized mice (colonized with human microbiota) consuming

a high-fat diet [204]. It tended to down-regulate both intestinal and systemic pro-inflammatory changes induced by the diet. A commercial preparation of bifidogenic growth stimulator (BGS), which is produced by *P. freudenreichii* ET-3, led to an improvement in the clinical activity scores of UC patients [205,206]. In the same study, patients also showed a decrease in the endoscopic index and an improvement in serum hemoglobin and albumin concentrations. Although, no clinical evidences on propionibacteria consumption within CRC patients exist, when tested in healthy men, this probiotic mixture reduced fecal α-glucosidase, which is associated with carcinogenesis [207,208]. Studies strongly suggest that those anti-inflammatory and potential anti-cancerous effects are related with the molecular factors of *P. freudenreichii* such as metabolites, S-layer proteins, short fatty acids, and 1, 4-dihydroxy-2-naphtoic acid [209–211].

4.3.1.1. S-layers proteins

S-layer proteins (Slps) constitute a surface-exposed proteinaceous lattice, non-covalently anchored to the cell wall via Surface Layer Homology (SLH) domains. This structure is present in many Gram-positive bacteria other than propionibacteria [212,213]. *P. freudenreichii* strains have seven genes encoding Slps proteins, exhibiting a wide variety of sequences between species but also within the same species [195]. S-layer proteins play various functions: adhesion, virulence factors, transport of molecules, masking of receptors to phages, and protection against environmental stresses [212,213]. The stimulation of Peripheral Blood Mononuclear Cells (PBMC) with *P. freudenreichii* Slps proteins mixture leads to the release of regulatory IL-10, in a dose-dependent manner. When applied in conjunction with a proinflammatory stimulus such as *Lactococcus lactis* MG1363 or *Escherichia coli* EPS, *P. freudenreichii* Slps considerably reduce the induction of the proinflammatory cytokines IL-12, IFN-γ and TNF-α [214]. The presence of a capsule of exopolysaccharides in several strains of *P. freudenreichii* blocks the immunostimulation of PBMCs, but the deletion of this EPS capsule by genetic mutation restores the immunomodulatory properties inthe mutant [215,216]. This indicates a key role of surface proteins as PAMPs in this probiotic/host cross-talk. A further molecular study specified that the immunomodulatory properties do not result from the presence of one single Slp protein but rather from a combination of several surface layer protein species [217].

4.3.1.2. Short-chain fatty acids

P. freudenreichii, among other dairy propionibacteria species, produces mainly acetate and propionate as SCFAs in ratio 2:1 by anaerobic fermentation of carbohydrates or organic acids [190]. Propionate and acetate were identified as responsible for the anti-cancerous effect of dairy propionibacteria in colorectal and gastric cancerous cells. The pro-apoptotic effect, confirmed in an animal model of carcinogenesis, was studied and the molecular mechanism

was determined [218–221]. SCFAs activate firstly the apoptotic intrinsic pathway, by acting on the mitochondria adenine nucleotide translator (ANT) pore. The ANT activation leads to mitochondria depolarization and permeabilisation; and then leakage of cytochrome C and caspase activation. Furthermore, as demonstrated by Cousin *et al.* (2016), SCFAs could act on the extrinsic apoptotic pathway by enhancing the cytotoxicity of the TNF-Related Apoptosis-Inducing Ligand (TRAIL) cytokine treatment in HT-29 cells [222] and by inducing expression of the corresponding R2/DR5 receptor, a TNF receptor super family member that mediates apoptosis by activating the extrinsic apoptotic death pathway. A combination lead to a modulation of genes expression involved in apoptosis, decreasing FLIPL and XIAP expression, which are two apoptosis inhibitors, regulating extrinsic and intrinsic cell death pathways respectively. SCFAs was demonstrated to have a Histone Deacetylase (HDACs) inhibitory activity in HT29 cells, which cause cell cycle arrest and p21 expression [222]. HDACs inhibition seems to be induced in part by SCFAs activated G-protein-coupled receptors, which are known to modulate gut immune system [223]. Finally, SCFAs treatments increased of NOD-like receptors and cytokine-cytokine receptors interaction gene expression, known to play a role in immune response [222]. Finally, *P. freudenreichii* consumption by humans increase SCFAs in feces, suggesting the possibility to modulate gut SCFAs concentrations for preventing CRC occurrence.

4.3.1.3. DHNA

Dairy probionibacteria, including *P. freudenereichii*, produce a vitamin K_2 (or menaquinone) biosynthesis intermediate, called 1,4-dihydroxy-2-naphtoic acid (DHNA) [224-226]. It is considered as bifidogenic component and modulates animal and human microbiota, in healthy and disease context. DHNA was shown to be able to stimulate *in vitro* and *in vivo* bifidobacteria growth. Indeed, the consumption of dried cultures of the *P. freudenreichii* ET-3 strain leads to an increased population of bifidobacteria within the human gut microbiota in healthy human volunteers [227,228]. Similar results were observed using a cell-free culture supernatant of *P. freudenreichii*, which was called bifidogenic growth stimulator (BGS) [229]. In addition, DHNA treatment was shown to restore Lactobacillus and Enterobacteriacea flora in dextran sulfate sodium (DSS)-induced-colitis in mice [230]. It induces also the expression of anti-microbial C-type lectin Reg III protein family, which certainly affects the microbiota [231]. DHNA is an anti-inflammatory metabolite which prevents inflammation in different murine colitis models [230–232]. It decreased the lymphocytes infiltration in tissues by reducing cell adhesion molecules expression (MAdCAM-1 or VCAM-1), in a mice colitis model [230,232]. Those adhesion molecules are highly expressed In IBD patients, which exacerbate the inflammation by increasing immune cells infiltration in tissues. DHNA acts via the aryl hydrocarbon receptor (AhR) activation, a transcriptional factor involved in inflammation [231]. AhR activation was shown to be involved in the inhibition of secretion of proinflammatory

cytokines. Indeed, the inhibition of proinflammatory cytokine IL6 in LPS-stimulated macrophages was related to AhR activation by DHNA [231].

5. Discussion

The Scientific community along with some enterprises have been through a technological race to sequence and characterize the genome of GIT commensal bacteria, the so-called gut microbiota. This approach is crucial to understand the interactions and associations within this high complexity biological system and with the host. The human intestinal microbiota composition is not only considered in the healthy state, but also in the context of disease, in order to understand the dysregulation of the cross talk mechanisms that are involved. Such dysregulation, especially when immune system is affected, lead to IBD, cancer or other inflammatory disorders, such as mucositis [7,18,19,46,64]. It is clear that the use of probiotics with anti-inflammatory or immunomodulatory properties, may change the microbiota composition, enhance epithelial barrier function and dampen immune responses by modulating inflammatory signaling pathways. Based on this rationale, several research groups aimed at treating gastrointestinal inflammatory disorders [103,127]. Due to adverse conditions of the GIT environment, it is important that probiotics be screened, in order to select tolerant strains to avoid massive bacterial death and loss of probiotic efficacy, while favoring robustness against digestive stresses, adherence to intestinal epithelium and long persistence in the host.

In this context, protection of probiotics could optimize fitness of sensitive strains or even improve tolerant strains, and consequently increase their beneficial effects in the GIT. Technological processes like microencapsulation, using biocompatible materials, or a combination of several processes that are used to make functional foods, have indeed been shown to enhance probiotic bacteria activity [182,233-235]. Currently, wide varieties of probiotics are available within commercial dairy products including fresh milk, yogurt and cheese. Interestingly, these commercial products may improve probiotics by converting biomolecules into dairy metabolites which can help in probiotic effect such as conjugated fatty acid, β-galactosidase enzyme, etc. [161,236,237]. For instance, fermented milk with *L. casei* BL23 showed a significant reduction of the clinical state of colitis in mice, suggesting that it is safe and efficient to use dairy fermented foods with probiotic strains in animal models [178]. In addition, this might be the initial step for their clinical use. Therefore, the search for new studies in different models of diseases should be encouraged [178,179].

Recent studies have pointed out the emergence of the potential probiotic application for *P.freudenreichii,* and other dairy propionibacteria, used extensively for Emmental cheese ripening, in the treatment of different gastrointestinal inflammatory diseases such as mucositis, colitis and in CRC using a rat model. In addition, ongoing studies investigate the benefit of designer fermented dairy products in the context of clinical trials (NCT02488954)

[104,105,187,188,238–240]. Finally, exploration of probiotic aptitudes in robust, traditional and easy-to-implement fermentation starter bacteria is a promising area of research.

6. Conclusion

The potential of different probiotic bacteria strains in treating GIT disorders, in animal models and in clinical trials, strongly suggests that they open avenues for the development of novel clinical biotherapies. We believe that the use of functional dairy foods is a useful way for enhancing immunological effects, as they provide additional beneficial properties and serve as excellent protection matrices for probiotic bacteria. In this context, exploring the potential of the variety of lactic acid and propionic acid bacteria selected by centuries of traditional fermentation worldwide, will allow identification of yet unknown superbugs.

7. Reference

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Chapter 2

Actinobacteria from less explored ecosytems: A promising source for anti TB metabolites

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Abstract

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a highly prevalent infectious disease with almost one-third of global population believed to be infected. According to statistics, India is one among the 22 high burden countries in terms of TB incidence rates. Emergence of drug resistance among *M. tuberculosis* isolates and long term therapy using combination of drugs for its treatment are the major problems in TB control. Hence, there is an urgent need for new antitubercular drugs to fight against drug resistant *M. tuberculosis* strains. These drugs are expected to have least side effects and improved pharmacokinetic properties with extensive and potent activity against drug resistant strains and/or should be able to reduce the total duration of treatment. Secondary metabolites from microbial sources have a long history in the treatment of TB. Actinobacteria - the group of Gram-positive filamentous bacteria, are the promising source for secondary metabolites which produce about one third of the antibiotics available in the market. Out of 33,500 microbial bioactive metabolites reported during 1940-2010, about 13,700 are reported to have been synthesized by actinobacteria in which most of them are from terrestrial origin. In recent years, actinobacateria from rare ecosystems have been recognised as most efficient groups of secondary metabolite producers with wide range of biological activities. This book chapter describes the TB burden and anti-TB agents that can be explored from actinobacteria which serves as a promising source.

1. Tuberculosis

Tuberculosis is a contagious airborne disease caused by the pathogen *Mycobacterium tuberculosis* (Mtb) [1]. According to World Health Organization (WHO), 9.6 million people are estimated to have fallen ill with TB in which around 123,000 patients developed Multiple Drug Resistance tuberculosis (MDR-TB) and 1.5 million died from the disease in year 2015. Thus TB poses a serious problem worldwide attributing to the increase in the rate of HIV-related TB, pediatric TB, latent TB, MDR- TB and Extensively Drug Resistant TB (XDR-TB). The real challenge has been to find a new drug effective against both replicating and non-replicating *M. tuberculosis*. Hence, there is a need for new molecules to fight against TB.

1.1. General characteristics of *M. tuberculosis*

Mycobacterium tuberculosis is a slow-growing, aerobic rod-shaped acid-fast bacterium (**Figure 1**). This bacterium is a highly contagious facultative intra-cellular parasite, usually of macrophages and has a slow generation time of 15-20 hours [2]. *M. tuberculosis* strains are very weak Gram-positive. Members of mycobacterial species contain a unique lipid-rich cell wall composition that allows them to take basic dyes and resist decolourization with acid-alcohol and so are called acid fast bacilli. Acid-fast bacilli appear pink against a blue back-ground when stained by Ziehl-Neelson staining. An agar based Middlebrook medium and egg based Lowenstein-Jensen (LJ) medium are the two important solid media commonly used to grow *M. tuberculosis*. The colonies are small and buff colored when grown on either medium. It takes 2-8 weeks to get visual colonies on either type of media. The cell wall structure of *M. tuberculosis* is unique among prokaryotes and it is a major determinant of virulence for the bacterium. The cell wall has high lipid content and allows the bacteria to survive within the macrophages. It also provides the organism with a resistant barrier to many common drugs [3, 4]. The cell wall mycolic acids are considered to be a significant determinant of virulence in *M. tuberculosis*. More complete understanding of the biosynthetic pathways and gene functions will enable the development of antibiotics to prevent formation of the cell wall which are areas of great interest [5,6].

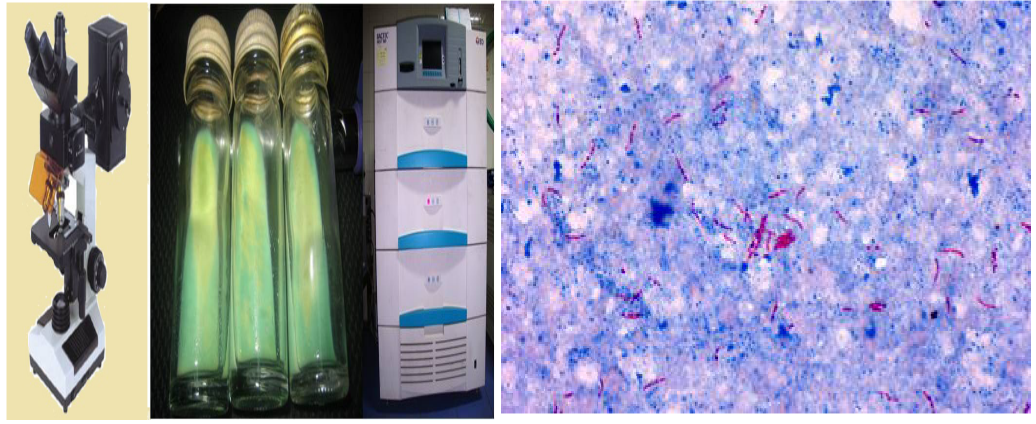


Figure 1: Cultural and micromorphology of *Mycobacterium tuberculosis*

1.2. Pathogenesis of tuberculosis

Human beings are the primary host for M. tuberculosis. Infection is spread via airborne dissemination of aerosolized bacteria containing droplet nuclei of 1-5 μm in diameter that carry *M. tuberculosis* from an individual with infectious TB disease to an uninfected individual. The infectious droplet nuclei are inhaled and get lodged in the alveoli in the distal airways. *M. tuberculosis* is then taken up by alveolar macrophages, initiating a cascade of events that result in either successful containment of the infection or progression to active disease (primary progressive TB) (**Figure 2**). Risk of development of active disease varies according to time since infection, age, and host immunity [7,8]. However, the life- time risk of disease for a newly infected young child has been estimated at 10%.

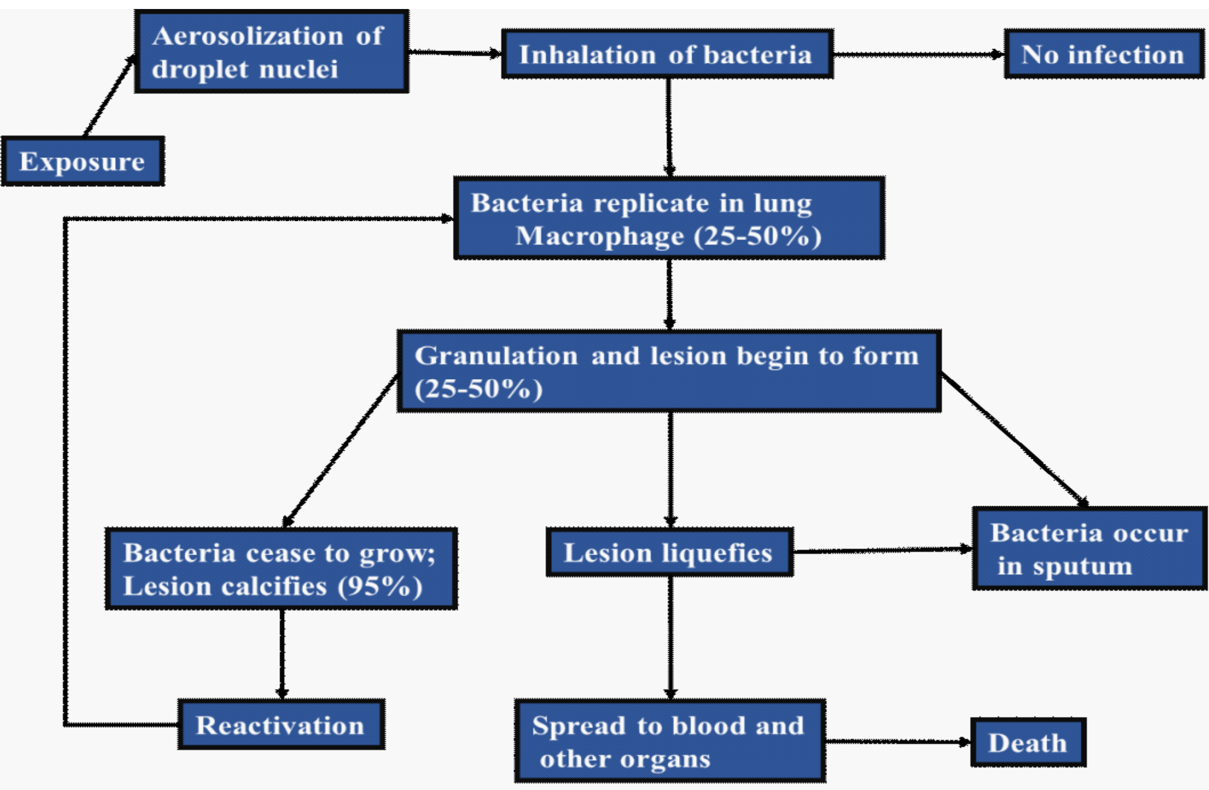


Figure 2: Pathogenesis of Tuberculosis

Latent tuberculosis infection (LTBI) is defined as a clinical condition without clinical or radiological signs of active disease and is manifested only by a positive tuberculin skin test [9]. Approximately 2 billion people or one third of the world's population, have LTBI, and approximately 10% of them will develop active TB during their life time. There is plenty of evidence that the basis for LTBI in humans is persistence of tubercle bacilli *in vivo* for long periods of time. This status is currently defined as dormancy or non-replicating persistence (NRP) [10].

1.3. Tuberculosis chemotherapy

Since the control measures for TB such as Bacillus Calmette Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, treatment by anti-TB drugs becomes

the only option available. The therapy of mycobacterial infections, in particular tuberculosis is challenging for a number of reasons. This bacterium is not susceptible to many classes of antibacterial agents. As a result, tuberculosis often requires treatment with drugs that are not commonly used for other microbial infections and often have small therapeutic windows.

The beginning of TB-chemotherapy illustrates well the importance of nature in the fight against diseases. The first drug discovered to treat TB-streptomycin (SM)-an aminoglycoside was isolated from the actinomycete, *Streptomyces griseus*, unlocking the door to the antibiotic treatment of TB [11]. While monotherapy with streptomycin was able to cure lethal forms of acute paucibacillary TB such as meningitis and miliary disease, it was soon evident that it resulted in the emergence of resistant mutants and treatment failure among patients with multibacillary forms like cavitary pulmonary TB. After the discovery of streptomycin, several synthetic drugs were introduced into the market. In 1946, Lehman from Sweden discovered para-aminosalicylic acid (PAS) as an effective TB drug. This was quickly followed in 1952 by the discovery of highly active TB drug isoniazid (INH). Both, PAS and INH ushered in the era of combination therapy. INH represented a major milestone in the tuberculosis chemotherapy because of its highly active, inexpensive nature with no significant side effect. Therapy with SM, PAS and INH prevented the selection of SM-resistant mutants and resulted in the cure of patients with 18 months of treatment. For more than 20 years this combination was the standard treatment for TB [12].

Remarkably, the nicotinamide led to the discovery of pyrazinamide (PZA) in 1952 and ethionamide (ETH)/prothionamide (PTH) in 1956. Further, screening of extracts from soil microbes led to the discovery of many other anti TB drugs viz. cycloserine, kanamycin and its derivatives such as amikacin, viomycin, capreomycin and rifamycins. Rifamycins and their derivatives are the drugs of choice for treatment of TB since 1970s [13,14].

The goals of tuberculosis treatment are to ensure cure without relapse, to prevent death, to impede transmission, and to prevent the emergence of drug resistance. Numerous antibiotics with anti-TB activity have long been classified as ‘first line’ or ‘second line’ drugs on the basis of their anti-TB activity and toxicity. First line drugs are with promising anti-TB activity and limited toxicity whereas the drugs with lesser activity and/or greater toxicity are considered as second line drugs (**Table 1**). Second-line drugs are used primarily in the treatment of patients harbouring bacilli resistant to the first-line drugs [15-18].

Table 1: Properties of first-line and second-line anti-TB drugs

Anti-TB drugs	Structure/class	Delivery route	Activity	Mechanism of action
First-line drugs				
Isoniazid	Pyridine hydrazide	Oral	Bactericidal	Inhibition of cell wall Mycolic acid synthesis and other multiple effects on DNA, lipids and NAD metabolism
Rifampin	Rifamycin	Oral	Bactericidal	Inhibition of RNA synthesis
Pyrazinamide	Nicotinamide analog	Oral	Bacteriostatic/ bactericidal	Disruption of membrane transport and energy depletion
Ethambutol	Ethylenediamine derivative	Oral	Bacteriostatic	Inhibition of cell wall arabinogalactan synthesis
Streptomycin	Aminoglycoside	IM injection	Bactericidal	Inhibits protein synthesis
Second-line drugs				
Kanamycin	Aminoglycooside	IM injection	Bactericidal	Inhibition of protein synthesis
Amikacin	Aminoglycoside	IM injection	Bactericidal	Inhibition of protein synthesis
Capreomycin	Polypeptide	IM injection	Bactericidal	Inhibition of protein synthesis
Para-aminosalicylic acid	Salicylic acid	Oral	Bacteriostatic	Inhibition of folic acid synthesis
Ethionamide	Thioamide	Oral	Bacteriostatic	Inhibition of cell wall Mycolic acid synthesis
Cycloserine	Isoxazolidinone	Oral	Bacteriostatic	Inhibition of cell wall Mycolic acid synthesis
Moxifloxacin	Fluoroquinolone	Oral or IV	Bactericidal	Inhibition of DNA replication
Gatifloxacin	Fluoroquinolone	Oral or IV	Bactericidal	Inhibition of DNA replication
Levofloxacin	Fluoroquinolone	Oral or IV	Bactericidal	Inhibition of DNA replication

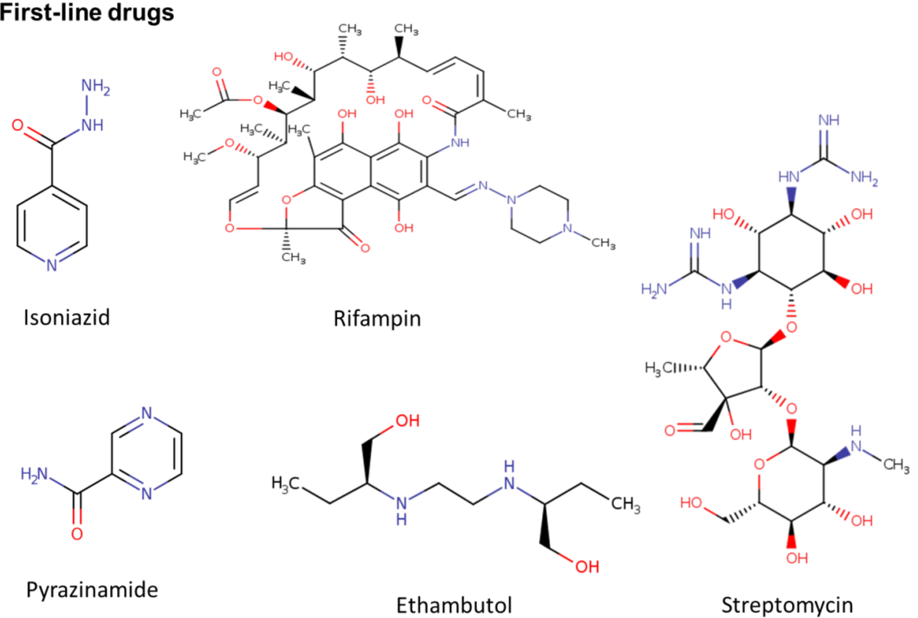


Figure 3: Structures of First line drugs used to treat drug-susceptible *M. tuberculosis*

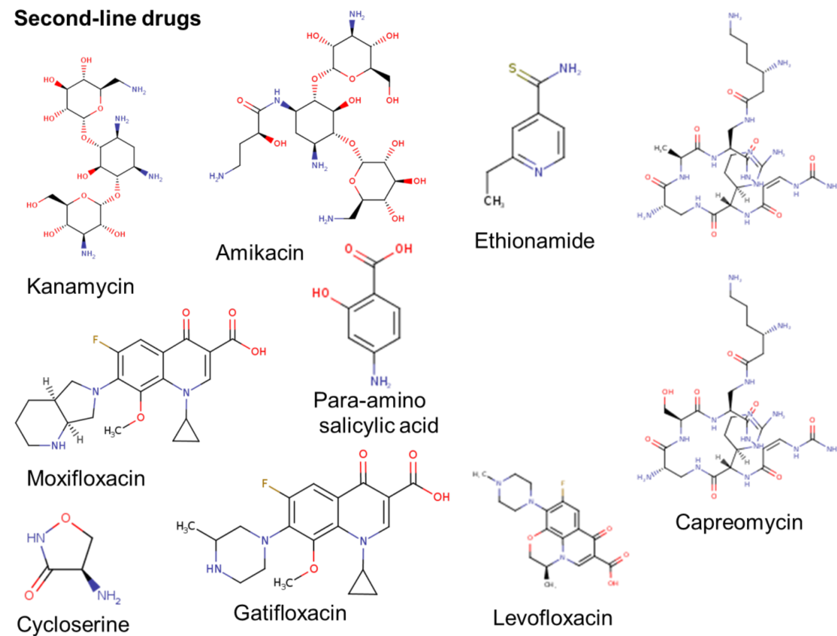


Figure 4: Structures of Second line drugs used to treat drug-susceptible *M. tuberculosis*

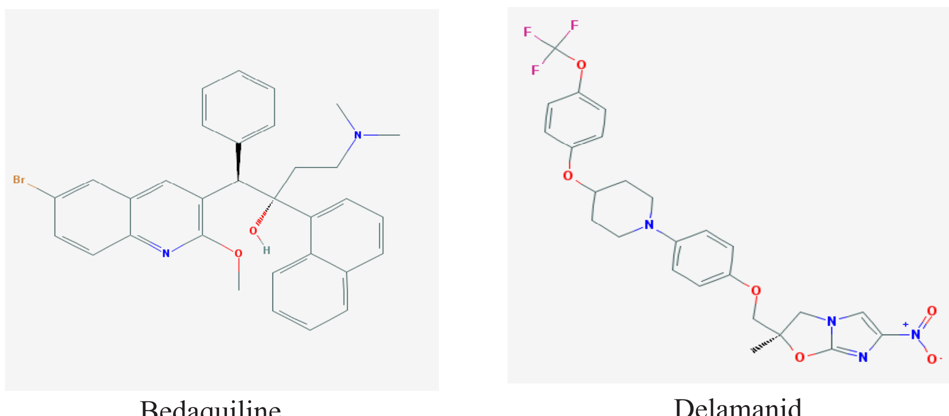


Figure 5: Recently Approved Drugs for the treatment of MDR-TB infections.

As suggested by WHO, the current standard chemotherapeutic regimen for treating new pulmonary TB patients consists of a multidrug combination of the first-line drugs comprising an initial intensive phase of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ or PZA), and ethambutol (ETB) daily for 2 months and a continuation phase of RIF and INH for a further 4 months, either daily or 3 times per week. INH and RIF are the two most potent anti-TB drugs that kill more than 99% of tubercular bacilli within 2 months of initiation of therapy [19, 20] . Using these drugs in conjunction with each other reduced the duration of anti-TB therapy from 18 months to 6 months.

Table 2: Recommended strategies for TB Therapy

	Regimen	Total duration (months)	Number of Drugs	Cost per patient
Susceptible TB	2 months INH+RIF+PZA+EMB followed by 4 months INH+RIF	6	4	USD 19-22
MDR TB Resistant pattern				USD 4000-6000 (ex works)
INH, RIF	PZA+EMB+FQN+1 SLD (entire course) + INJ (first 6 months)	18-24	5-6	
INH, RIF (EMB/ PZA)	(PZA or EMB) + FQN + 2 SLD (entire course) + INJ (first 6 months)	18-24	5-6	
INH, RIF, EMB, PZA	FQN + 3 SLD (entire course) + INJ (first 6-12 months)	18-24	5-7	
INH, RIF, EMB, PZA (FQN/IN)	(INJ or FQN) + 3 SLD + 3TLD (entire course)	>24	5-7	
INH, RIF, EMB, PZA, FQN, INJ	INJ + all available SLD +TLD (entire course)	>24	5-7	

Resistance pattern is based on the results of drug susceptibility tests.
Abbreviations: INH: isoniazid; RIF: rifampicin; PZA: pyrazinamide; EMB: ethambutol; FQN: fluoroquinolones; INJ: injectable drugs (eg. Streptomycin, kanamycin); SLD: second line drugs; TLD: third line drugs (eg. Clarithromycin, amoxicillin, linezolid); All treatment administrations are performed on a daily basis

1.4. Limitations of current tuberculosis therapy

When administered appropriately, combination anti-TB therapy can be highly effective any where in the world. Regimens employing first-line drugs are orally bio-available, relatively cheap, and generally well tolerated. But this regimen is lengthy and complex, inviting non-adherence, drug interactions, drug toxicity, and treatment programs require substantial supervision to monitor adherence and tolerability [21]. New regimens for TB that could be administered for a shorter duration of therapy or more intermittently without sacrificing efficacy would reduce the burden of supervising drug administration and make treatment more widely available. Unfortunately, it is difficult to see how existing first-line drugs could be used more effectively in this regard, and there are no new agents in the later stages of the drug development pipeline [22]. In addition, the emergence of *M. tuberculosis* strains to the available drugs causes major concern which results in higher death rates, especially among HIV-infected persons.

Further, pediatric patients constitute a high risk population by *M. tuberculosis* infection. According to WHO statistics, 250,000 children develop the disease and approximately 100 000 die every year. Pharmacokinetics of several anti-TB drugs has shown poor efficacy in children. Also, there are a very limited number of anti-TB liquid formulations. Most of the first-line drugs are not available in pediatric form and they are produced only extemporaneously. For example, a liquid suspension of RIF (Rifaldin®, Sanofi-Aventis) is available in Spain. This

not only results in less compliant regimen but also makes dose-per-body weight adjustments difficult. Manipulation of solid forms (e.g. crushing and mixing with food or beverages) may lead to unpredictable changes in the stability of active compounds and their bioavailability.

1.5. Anti-TB drug resistance

The history of anti-TB drug resistance is fairly recent, emerging just over 60 years ago with the development of anti-TB drugs. For decades, the problem was identified in local areas among patients treated at reference centres in industrialized countries. With the discovery of rifampicin in 1966, and the expansion of its use between 1970 and 1990, patients who were already carriers of isoniazid resistant to *M. tuberculosis* strains became resistant to rifampicin. This was the start of a progressively growing problem which has reached epidemic proportion in some countries [23]. An individual may develop the drug resistant form of TB via inadequate therapy that enable the selection of drug resistance (acquired resistance) or infection with a drug-resistant TB strain (primary resistance).

Drug resistance in *M. tuberculosis* mostly occurs as a result of man-made selection during disease treatment. Resistance in *M. tuberculosis* develops through a limited number of mechanisms at low frequency. Mutations in the enzymes that either activate antimycobacterial drugs or are targets of drug action most commonly associated with drug resistance. Drug inactivation mechanisms that result in resistance has been of limited clinical interest because such compounds are not used in the treatment of tuberculosis. Limited number of drug efflux mechanisms has yet been described that can account for drug resistance in *M. tuberculosis*, although diffusion and transport into mycobacterial cells is an extremely important variable in drug activity. Role of these efflux pumps in clinical scenario in different patient population is not yet completely defined or explained. Episomal or transposon-mediated transfer of resistance genes into *M. tuberculosis* has not been demonstrated till date, though this is a common mechanism for the acquisition of drug resistance in other bacteria. The biochemical transformations occurring in mycobacteria during the acquisition of drug resistance are generally inferred, rather than demonstrated, and can be identified as thrust area for research in future.

The emergence of MDR-TB and XDR-TB pose serious threat to the public and further complicates the TB global emergency. They are resistant to the best antibiotics and are associated with greater morbidity and mortality than antibiotic susceptible TB. While infection with an exogenous drug resistant TB strain is related to infection control measures, the development of acquired *M. tuberculosis* is multi-faceted and can be attributed to various social, political, economic, epidemiological and pathophysiological factors [19,24]. Efforts to understand the molecular basis of *M. tuberculosis* antibiotic resistance have advanced significantly and investigations of potentially unique genetic traits in MDR- and XDR-TB strains are ongoing. Unlike other bacterial pathogens, there is no evidence that gene acquisition contributes to an-

tibiotic resistance in *M. tuberculosis*.

While MDR-TB can be effectively treated with a long-term regimen of second-line antibiotics, XDR-TB is often considered very difficult to treat, or is even untreatable, with existing chemotherapeutic agents. The diagnosis of MDR-TB or XDR-TB further subjects the patients to as many as 20 pills per day, as well as antibiotic intramuscular injections for 18-24 months. This lengthy treatment is not only more expensive than first-line antibiotics, but also comes with devastating, toxic side effects, emotional and social anxieties and psychological stresses. A large retrospective study revealed that XDR-TB cases have a worse clinical outcome than MDR-TB cases resistant to all first-line antibiotics (39% vs 54% treatment success, respectively) [19,20,25]. Therapy for LTBI is also protracted and comes in various regimens that may contain any combination of isoniazid, rifamycin, pyrazinamide and an approved fluoroquinolone, in the case of drug resistant LTBI. In order to combat MDR and XDR-TB and the overall spread of antibiotic resistant TB strains, the need for new anti-TB antibiotics is imminent. A better understanding of the mechanisms of action and development of drug resistance will allow identifying new anti-TB drug targets and better ways to detect drug resistance [26].

Table 3: Anti-TB drugs and their resistance mechanisms

Anti-TB drugs	Mechanism of action	Mechanism of resistance
Isoniazid	Inhibition of cell wall Mycolic acid synthesis and other multiple effects on DNA, lipids and NAD metabolism	KatG suppression causing decreased prodrug activation, and a mutation in the promoter region of InhA causing an overexpression of InhA
Rifampin	Inhibition of RNA synthesis	Mutation of rpoB induces a conformational change at β-subunit of RNA polymerase causing a decrease in binding affinity
Pyrazinamide	Disruption of membrane transport and energy depletion	Mutations in pncA reducing conversion to active acid form
Ethambutol	Inhibition of cell wall arabinogalactan synthesis	Mutations in embB at codon embB306
Streptomycin	Inhibits protein synthesis	Mutations in rpsL and rrs confer binding site modulation
Amikacin/ Kanamycin	Inhibition of protein synthesis	16S rRNA target site modulation (1400 and 1401 rrs gene) Increased drug inactivation via overexpression of eis aminoglycoside acetyltransferase
Capreomycin	Inhibition of protein synthesis	Cross-resistance with aminoglycosides plus mutation of tlyA which decreases rRNA methyltransferase activity
Para-aminosalicylic acid	Inhibition of folic acid synthesis	Mutations in the thyA causing a decrease in activated drug concentrations and folC mutations which cause binding site mutations

Ethionamide	Inhibition of cell wall Mycolic acid synthesis	Mutations in ethA and inhA causing decreased prodrug activation and InhA mutations which cause binding site mutations
Cycloserine	Inhibition of cell wall Mycolic acid synthesis	Overexpression of alrA decreasing drug efficiency
Moxifloxacin/ Gatifloxacin	Inhibition of DNA replication	Chromosomal mutations in the quinolone resistance-determining region of gyrA or gyrB. The most frequent mutations found are at position 90 and 94 of gyrA

1.6. Global portfolio of candidate anti-TB drugs in clinical development

After the discovery and development of new anti-TB drugs flourished in the mid-1900's, the TB drug pipeline was reduced to a mere leaky faucet with the new classes of antibiotics virtually nonexistent. It has been more than 40 years since the last novel TB-specific antibiotics were introduced into clinical practice. Most of the drugs being used to treat tuberculosis were discovered before 1950s. However, recently the US FDA approved bedaquiline for MDR-TB and delamanid as a compassionate care option for XDR-TB and TDR-TB infections. The fact is that these drugs have pronounced issues, including hERG toxicity concerns, as well as multiple ADME issues due to their high lipophilicity. It is mandated that bedaquiline has to be used only in patients who do not have other treatment options [13,16,27-29].

Given the challenge of treating MDR and XDR-TB, there are some new classes of antibiotics in the current anti-TB pipeline. There are at least 20 drugs in various stages of clinical evaluation for TB till June 2017. These can be divided into several categories: i) novel drugs being developed for TB treatment, ii) current first line TB drugs being re-evaluated to optimize their efficacy and iii) currently licensed drugs for other indications and next /generation compounds of the same chemical class being re-proposed for TB (**Table 4**).

Table 4: Drugs in clinical evaluation for tuberculosis

Pre-clinical		Clinical		
Early stage development	**GLP Tox.**	**Phase 1**	**Phase 2**	**Phase 3**
CPZEN-45 SATB082 Spectinamide-1810 SPR-720 (pVXc-486) TBI-166 TBI-223 TB-47	BTZ-043 GSK-070 TBA-7371 TBAJ-587	OPC-167832 PBTZ169 Q203	Delpazolid (LCB01-0371) SQ-109 Sutezolid	Bedaquiline (TMC-207) Delamanid (OPC-67683) Pretomanid (PA-824)

Table 5: Required properties of new anti-TB antibiotics

What a new antibiotic should do	Characteristic(s) required
Simplify treatment or reduce treatment duration	Strong (early) bactericidal and sterilizing activity Low pill count, fixed dose combinations Allow for intermittent therapy
Have an acceptable toxicity profile	Low incidence of treatment-limiting adverse events No overlapping toxicity profile with other TB drugs
Be active against MDR/XDR TB	No cross-resistance with first-line drugs
Be useful in HIV infected patients with TB	Minimal interactions with antiretroviral drugs No overlapping toxicity profile with antiretroviral drugs
Be active against latent TB	Activity against dormant bacilli Favourable toxicity profile

1.7. Anti-TB natural products

Although different types of anti-TB agents are available in world market, there is a growing interest in natural products for novel anti-TB drug discovery, due to non-specific side effects associated with synthetic therapeutics agents and unusual chemical diversity present in natural products. Natural products have been recognized as the source of most active ingredients of medicine. More than 80% of drug available in world market were natural products or inspired by them. Natural products derived scaffolds are therapeutic templates for the design of new therapeutic drugs using medicinal chemistry and computer-assisted design techniques. Thus, they have a remarkable impact on the treatment of TB in comparison with classical FDA-approved drugs such as rifampicin, kanamycin and cycloserine. Anti-TB compounds isolated from natural sources such as plants, microbes and marine organisms have been found with different skeleton chemical forms and conformations [30,31].

Plants have been used worldwide in traditional medicines for the treatment of various diseases and it is estimated that even today approximately 65-75% of the world's population rely on medicinal plants as the primary source of medicines. The phytochemical study of some of these plants has yielded a number of active natural products. Next to microorganisms, plants are the important source for anti-TB compounds. Several recent review and research articles have highlighted the underutilized potential of plant species as sources of antimycobacterial extracts and chemicals [32-34]. Among the plant-derived antimycobacterial compounds belonging to an exceptionally wide diversity of classes, alkaloids, terpenoids, coumarins, peptides and phenolics are more dominant. Of 17,500 higher plant species occurring in India only about 365 species have been evaluated so far for antimycobacterial activity [32,35].

The potential of marine organisms is well documented in the recent past. Yet, their utility for anti-TB drug discovery is still in its infancy. Till 2000, there are only two reports of *in-vitro* anti-TB activity from marine origin. Massetolide A and viscosin are cyclic depsipeptides isolated from cultures of *Pseudomonas* species isolated from a marine alga and tube worm, respectively. There are very few anti-TB compounds isolated from marine macro

organisms such as molluscs (kahalalides A and F), sponges (heteronemin), corals (litosterol) [36,37]. Bioactive substances from natural sources are available in extremely low quantities leading to limitations in using the reservoir of marine organisms for bioassay and therapy. To overcome these problems, few methodologies such as mariculture, bioreactors, sponge cell culture, genetic modification and most importantly chemical and semi-synthetic approach can be pursued. Certain anti-TB compounds produced by marine sponges (agelasine) and corals (litosterol) have been synthesized by chemical methods [38]. Unfortunately, none of the several hundreds of non-microbial natural products with antimycobacterial activity have moved forward in drug development.

Microorganisms that live together in the environment develop long-lasting methods to keep each other at bay. As a result, many of our most effective bactericidal agents have come from environmental organisms. Microbes are the most exploited sources for bioactive natural products including anti-TB compounds. Till date, more than 1000 antimycobacterial compounds have been reported from microbial sources among which actinomycetes are the best reported microbial source. The entire commercially available natural product based-anti-TB drug in current practice is only from actinomycete origin. There are very few reports on anti-TB compounds from other bacteria such as *Janthinobacterium* sp. Ant5-2 (J-PVP) and *Flavobacterium* sp. Ant342 (F-Y OP) isolated from land-locked freshwater lakes of Schirmacher Oasis, East Antarctica and *Bacillus subtilis* isolated from leaf of eggplant, China [39,40].

1.7.1. Actinobacteria

Actinobacteria are aerobic, gram positive filamentous bacteria with high G+C (Guanine+ Cytosine) containing DNA. Actinobacteria was first discovered by Ferdinand Cohn in 1875 and it was first named by Actinomyces (ray fungus) by Harz in 1877. Actinobacteria was first recognized by Gasperini in 1890 as potential destroyers of bacteria and fungi [41]. Actinobacteria grow well on simple laboratory media with different chemical composition but their growth is much slower than that of other bacterial groups. In solid medium, most actinobacteria form leathery, smooth surfaced, cottony colonies with varying sizes. Most actinobacteria genera form mycelial growth called substrate/vegetative or primary mycelium. In addition, from the primary mycelium, the secondary/aerial or reproductive mycelium grows on the surface of the medium which form asexual spores. The temperature ranging between 20^{0}C and 30^{0}C and p^{H} between 5.5 and 8.0 are conducive for the growth of most actinobacteria. Nearly one month of incubation is needed for the primary isolation of actinobacteria [42-44].

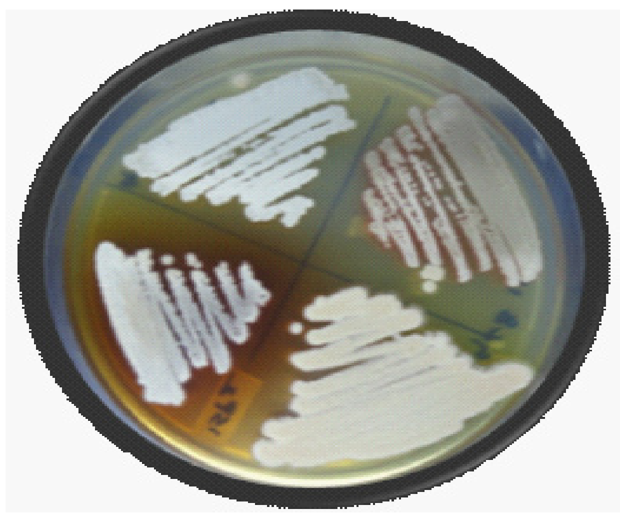


Figure 6: Cultural morphology of actinobacteria on ISP2 agar medium

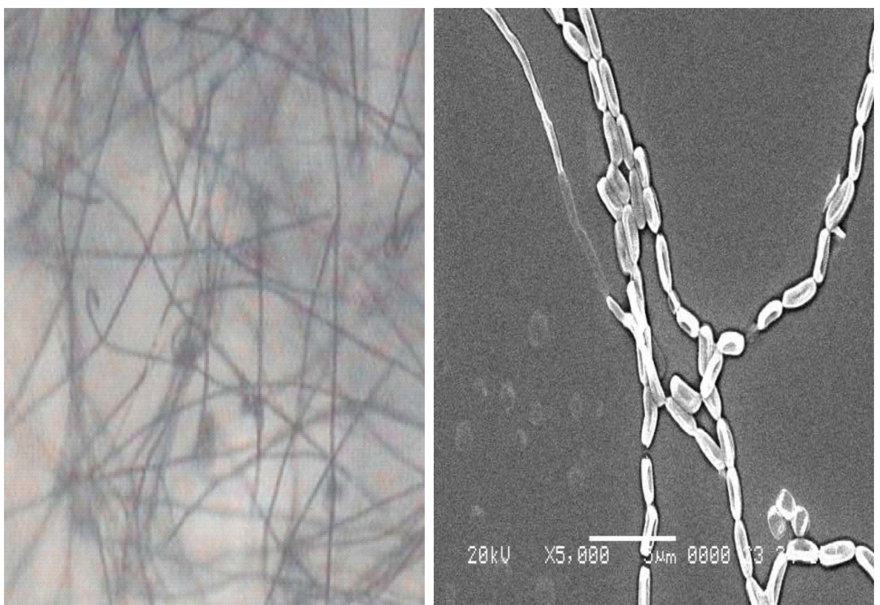


Figure 7: Micromorphology of actinobacteria (Streptomyces)

Actinobacteria are the most successful group of bacteria that occur in multiplicity of natural and man-made environments due to their ability to utilize all the available substrates in the environment. They are present in both terrestrial and aquatic ecosystems. Most of the Actinobacteria are free living saprophytes but some are parasitic or symbiotic to plants and animals. Soil is the single most reservoirs for actinobacteria. Among the total microbial population in soil, actinomycetes group occupies 10 to 50%. A single gram of rich agriculture soil can contain 10^6 colony forming units (CFU) of Streptomyces and 10^4-10^5 CFU of *Micromonospora* and other genera. The number and types of actinomycetes are highly affected by the physico-chemical properties of soil, climatic condition of that particular ecosystem, etc., [45,46].

1.7.2. Bioproducts from actinobacteria

Amongst prokaryotes, members of the order Actinomycetales, notably the genus *Streptomyces*, remain the richest source of natural products, including clinically useful antibiotics,

antimetabolites, antiparasitic, antiviral and antitumor agents [47,48]. Actinobacterial sources account for about 45% of all microbial bioactive secondary metabolites with 7600 of these compounds (80%) being produced by Streptomyces. About 74% of all actinobacteria products and 70-75% of various bacterial products exhibit antibacterial and/or antifungal activities. In contrast, only 40-45% of all fungal products have some kinds of antimicrobial activity against fungi. The antitumor activity is displayed by 30%, 24% and 27% of actinobacterial, bacterial and fungal products, respectively [47]. Despite this astonishing productivity, it has been predicted that only about 10% of the total number of natural products that can be synthesized by these organisms have been discovered [49]. The application of genomic technologies which showed that the whole genomes of *Rhodococcus* sp. RHA1, *Saccharopolysporaerythraea* NRRL 23338, *Salinisporatropica, Streptomyces avermetilis* MA-4680 and *Streptomyces coelicolor* A(3)2 contained around 20 or more natural product biosynthetic gene clusters for the production of known or predicted secondary metabolites [50-52].

The power of actinobacteria in the competitive world of chemical synthesis can be appreciated by the fact that even simple molecules are made by fermentation rather than by complex chemical synthesis. Most of the actinobacterial natural products are so complex and contain many centres of asymmetry that they will probably never be made commercially by total organic synthesis. There are five major groups of bioproducts produced by actinobacteria [53]. This includes primary metabolites, secondary metabolites, bioconversion products, microbial cell products and recombinant products (**Table 6**). Actinobacterial genera such as *Streptomyces, Rhodococcus* and *Thermomonospora* are recognized as a new source for the biosynthesis of gold and silver nanoparticles. Actinobacteria are also employed in the biodegradation of complex environmental pollutants [42,43,54,55].

Table 6: Major groups of bioproducts produced by actinobacteria

Group and Types	Some examples	Producers
Primary Metabolites		
Enzymes	Protease	*Streptomyces hygroscopicus*
	Lipase	*S. levendulae*
	Amylase	*Thermomonosporasp*
	L-asparaginase	*S. plicatus*
	L-glutaminase	*Streptomyces sp.*
Vitamins	Vitamin B_{12}	*S. olivaceus*
Amino acids	L-phenyl alanine	*Rhodococcus sp.*
	Lysine	*Nocardiaalkaloglutinosa*
Nucleotide	Guanosine	*S. griseus*
Siderophores	Madurastatin	*Actinomaduramadurae*
	Desferrioxamine B	*S. griseus*

Secondary metabolites		
Antibacterial	Streptomycin	*S. griseus*
	Chloramphenicol	*S. venezulae*
	Tetracycline	*S. rimosus*
	Erythromycin	*Saccharopolyspo-raerythraea*
	Rifampicin	*Amycolatopsismedit-teranei*
	Gentamicin	*Micromonosporaechi-nospora*
Antifungal	Amphotericin B	*S. nodosus*
	Candicidin	*S. griseus*
Antiviral	Fattiviracin	*Streptomyces sp.*
Insecticide and antiparasitic	Avermectin	*S. avermetilis*
	Milbemycin	*S. hygroscopicus*
Anticancer	Actinomycin D	*S. antibioticus*
	Mitomycin D	*S. levendulae*
Anticholestrolemic	Pravastatin	*S. carbophilus*
Growth promoter	Monensin	*S. cinnamomiensis*
	Tylosin	*S. fradiae*
Herbicide	Bialophus	*S. gradiae*
Immmuno Suppres-sive	Rapamycin	*S. hygroscopicus*
	Tacrolimus (FK506)	*Streptomyces sp*
Pigments	Actinorhodin	*Streptomyces sp.*
	Prodigiosin	*Streptoverticillium sp.*
Bioconversion products		
Antibiotic	Tetracycline	*S.aureofaciens ATCC10762*
	Streptomycin-P	*S. griseus*
Amino acid	Phenyl acetic acid	*Nocardiasp*
Polysaccharide	Chitosan	*Streptomyces sp.*
Amides	Acrylamide	*Rhodococcussp*
Microbial cell products		
Biofertilizers		*Frankiasp*
Biocontrol agents		*S. fradiae*
SCP [in animal feed]		*Thermomonosporafusca*
Recombinant products		
Antibiotics	Dihydrogranaticin	*Streptomyces sp*
	Miderrhodine A	*Streptomyces sp*

Bioactive metabolites, in particular antibiotics, production by actinobacteria is strain-specific and conditional. It has long been known that there are actinobacterial strains belonging to the same species that produce antibiotics different from one another and also that there are strains belonging to different species that produce the same antibiotic. Antibiotic production is therefore, not species specific, but strain specific [56,57].

1.7.3. Anti-TB compounds from actinobacteria

The first drug discovered to treat TB was streptomycin, an aminoglycoside isolated from the actinomycete *Streptomyces griseus*. Another outstanding example is rifampicin, an ansamycin antibiotic isolated from *Streptomyces mediterranei* renamed as *Amycolatopsismediterranei*. Following the discovery of streptomycin, in the period known as the golden era of TB research (1940-70), several synthetic drugs were introduced in the market. However, actinomycetes still played a crucial role in drug discovery against TB. For example, other aminoglycosides such as kanamycin from *Streptomyces kanamyceticus*, the semi-synthetic amikacin produced from kanamycin A and capreomycin from *Streptomyces capreolus*, as well as D-cycloserine from *Streptomyces* sp., are being used in TB treatment as second line drugs [58,59].

Some important anti-TB compounds isolated from actinomycetes are given in **Table 7**.

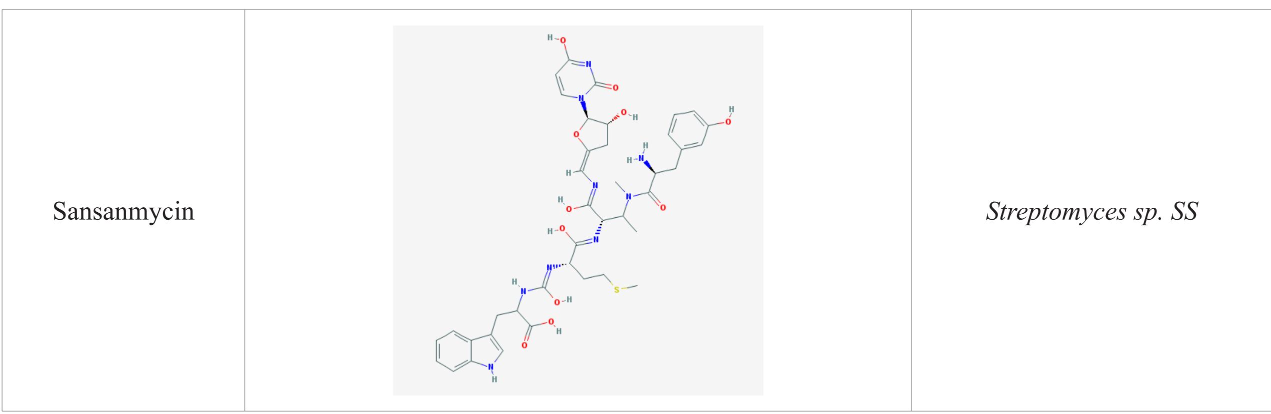
Table 7: Anti-TB antibiotics reported from actinomycetes

Anti-TB antibiotics	Chemical Structure	Producing actinomycetes
Actinomycin		*Actinomyces sp.*
Streptothricin		*Streptomyces sp*

Streptomycin		*Streptomyces griseus*
Nocardicin	b	*Noardiacoeliaca*
Neomycin		*Streptomyces fradiae*
Aureomycin		*Streptomyces aureofaciens*
Viomycin		*Streptomyces puniceus*

Elaiomycin		*Streptomyces gelaticus*
Tetracycline		*Streptomyces rimosus*
Cycloserine		*Streptomyces orchidaceus*
Kanamycin		*Streptomyces kanamyceticus*
Streptovaricin		*Streptomyces spectabilis*
Acetomycin		*Streptomyces ramulosus*
Tubermycin		*Streptomyces misakiensis*

Questiomycin		*Streptomyces sp*
Capreomycin		*Streptomyces capreolus*
Rifamycin		*Amycolatopsismediterranei*
Tuberactinomycin		*Streptomyces griseoverticillatus*
Paramomycin		*Streptomyces sp*
Pamamycins		*Streptomyces alboniger*



Sansanmycin		*Streptomyces sp. SS*

1.7.4. Actinobacteria from rare/less explored ecosystems

Nowadays, it is becoming increasingly difficult to find novel metabolites from common actinomycetes as regular screening leads to the rediscovery of mostly known compounds. However, it is not the end of an era but an endless frontier. Using standard procedures for the isolation of novel actinomycetes from poorly studied habitats is an alternative [49] and by applying new methods, rare or uncommon actinomycetes can be isolated. Novel species may contain unique compounds as the evolution of secondary metabolites [60].

Actinobacteria are traditionally considered as organisms that cannot occupy natural ecological niches that are characterized by extreme conditions. Actinobacteria have specific environmental needs differing from those of other mycelial bacteria. Now-a-days, great amount of data on the isolation of actinobacteria resistant to extreme environmental factors like acidity, salinity, temperature and pressure has been accumulated. Actinobacteria are isolated from various rare ecosystems such as forests, mountains, deep sea, desert and alkaline soils all over the world [61-63] (**Table 8**). But there are very few reports on antitubercular compounds from actinobacteria of rare ecosystems.

Table 8: List of some novel actinobacteria isolated from rare ecosystems

Organisms	Source	Special features
Beutenbergia cavernae gen. nov. sp. nov.	Cave soil, China	-
Nocardiopsiskunsanensissp.nov	Saltern, Kunsan Republic of Korea	Halophilic
Actinopolymorphasingaporensis gen. nov. sp. nov	Tropical forest soil, Singapore	Halophilic
Nocardiopsishalotolerans sp. nov	Salt marsh soil, Kuwait	Halophilic
Nocardiopsismetallicus sp. nov	Alkaline slag dump, Germany	Alkaliphilic
Nocardiopsisalkaliphila sp. nov.	Soil from Estern Desert, Egypt	Alkalophilic
Nocardiopsisaegyptia sp. nov	Marine sediments, Abu Qir Bay, Egypt	Halophilic
Salinispora arenicola gen. nov. sp. nov.	Marine sediment	Obligate marine actinomycete
Nocardiopsisarabia sp. nov.	Sand Dune soil, Egypt	Halotolerant
Nocardiopsisvalliformis sp. nov	Alkali lake soil, China	Alkaliphilic

Gracillibacillushalophilus sp. nov	Saline soil, Qaidam Basin, China	Halophilic
Saccharopolysporaqijiaojingensis sp. Nov	Salt lake, North west China	Halophilic
Amycolatopsis marina sp. nov	Ocean sediment of South China Sea	Halophilic
Nocardiopsis terrae sp. nov.	Saline soil, China	Halophilic
Streptomyces sannunensis sp. nov.	WadiSannur, Egypt	Alkalophilic
Yahishiella deserti gen. nov. sp. nov.	Desert soil,	
China	Thermotolerant	
Verrucosisporawenchangensis sp. nov.,	Mangrove soil, China	-
Thermoactinospora rubra gen. nov. sp. nov.,	Tengchong National volcanic geological park, China	Thermophilic

1.7.5. Anti TB activity of actinobacteria isolated from rare/less explored sources

More compounds from actinobacteria of terrestrial and marine origin are still in different stages of investigation to be developed as potential anti-TB drugs. Some of the reports on anti TB activity of actinobacteria from rare / less explored ecosystems are described below.

Cyclomarin A, a novel anti-inflammatory cyclic peptide, is one of three cyclomarin compounds isolated from marine *Streptomyces* spp. CNB-982. Recently, cyclomarin A was revived as an anti-TB lead compound based on results of whole cell-based screening assays [64]. Cyclomarin A is composed of seven amino acids, of which two are common amino acids (alanine and valine), and five are unusual amino acids [N-methylleucine, N-methylhydroxyleucine, β-methoxyphenylalanine, 2-amino-3,5-dimethylhex-4-enoic acid, and N-(1,1-dimethy l-2,3-epoxypropyl) β-hydroxytryptophan] [65].

In another study, crude bioactive compounds from 15 actinobactreial strains isolated from rare marine and forest ecosystems was produced by shake flask fermentation using soybean meal medium. Culture supernatant and mycelia were extracted with ethyl acetate and methanol, respectively. Antibacterial activity of crude extracts was tested by disc diffusion method against gram positive and gram negative bacteria. Actinobacterial strains D10, D5, NEK5, ANS2, M104 and R2 showed prominent activity. Culture filtrates and crude extracts were tested against standard strain Mycobacterium tuberculosis H37Rv and drug sensitive and drug resistant clinical isolates of *M. tuberculosis* by luciferase reporter phage (LRP) assay. Considerable variation was observed in antimycobacterial activity between actinobacterial culture filtrates and solvent extracts. Actinobacterial strains viz., D10, D5 (desert), CSA14 (forest), CA33 (alkaline soil), NEK5 (Neem plant), MSU, ANS2, R2 and M104 (marine) screened in the present study were found to be highly potent showing good antibacterial and antimycobacterial activity. Five of them such as A3, CSA1, EE9, ANS5 and R9 were exclusively active against *M. tuberculosis*. Secretary products of actinobacteria of rare ecosystems are meant to antagonize organisms in their respective environments. These are likely to be novel antimycobacterial compounds as they unknown to human pathogens [66].

Crude bioactive comounds produced from 14 facultative psychrophilic actinobacteria were screened for antimycobacterial activity against *Mycobacterium tuberculosis*. The results showed that, four strains were active against the test organism *M. tuberculosis*. The active isolates screened in the present study were found to be highly effective and comes under Streptomycetes species (RH7 and RH8), *Micromonospora* species (RH9) and *Micropolyspora* species (RH12). All the isolates capable of producing metabolites and their presence were confirmed by TLC. The 'active culture filtrate' showed 'only one band' and its functional group is low molecular weight neutral compounds and amines were determined based on their solubility and pH [67].

Bioactive potential of actinobacteria isolated from certain less explored Indian ecosystems was tested against *Mycobacterium tuberculosis* and other non-mycobacterial pathogens. Actinobacteria were isolated from the soil samples collected from desert, coffee plantation, rubber forest, and hill area from Western Ghats and Eastern Ghats Ecosystems in India and their cultural and micro morphological characteristics were studied. Crude extracts were prepared by agar surface fermentation and tested against *M. tuberculosis* isolates by luciferase reporter phage (LRP) assay at 100 μg/mL. Activity against non-mycobacterial pathogens was studied by agar plug method. Totally 54 purified cultures of actinobacteria including 43 *Streptomyces* and 11 non *Streptomyces* were isolated. While screening for antitubercular activity, extracts of 39 actinobacteria showed activity against one or more *M. tuberculosis* isolates whereas 27 isolates exhibited antagonistic activity against non mycobacterial pathogens. In particular crude extracts from sixteen actinobacterial isolates inhibited all the three *M. tuberculosis* isolates tested. Findings of the present study concluded that less explored ecosystems investigated in this study are the potential resource for bioactive actinobacteria. Further purification and characterization of active molecule from the potential extracts will pave the way for determination of MIC, toxicity, and specificity studies [68].

Five new nucleoside antibiotics, named streptcytosines A–E, and six known compounds, de-amosaminyl-cytosamine, plicacetin, bamicetin, amicetin, collismycin B, and SF2738 C was isolated from a culture broth of *Streptomyces* sp. TPU1236A collected in Okinawa, Japan. The structures of new compounds were elucidated on the basis of their spectroscopic data (HRFABMS, IR, UV, and 2D NMR experiments including ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY spectra). Streptcytosine A belonged to the amicetin group antibiotics, and streptcytosines B–E were derivatives of de-amosaminyl-cytosamine, 2,3,6-trideoxyglucopyranosyl cytosine. Streptcytosines A inhibited the growth of *Mycobacterium smegmatis* (MIC=32 μg/mL), while compounds streptcytosines B-E were not active at 50 μg/disc. Bamicetin and Amicetin showed the MICs of 16 and 8 μg/mL, respectively [69].

The feasibility and relevance of screening a library of raw actinomycete extracts (ECUM library) for the identification of antituberculosis activities was assessed on 11,088 extracts us-

ing a multiple-screening approach. Each extract was first tested at two concentrations against non-infected macrophages as a control, then against *Mycobacterium tuberculosis* growing in broth medium as well as infecting murine macrophages. The screening results indicated a library of good quality with an apparent low proportion of cytotoxic extracts. A correlation was found between both bacterial assays, but the intracellular assay showed limitations due to low rates of cell survival. Several extracts of interest were highlighted by this multiple screening. A focus on the strain producing the two most effective revealed similarities with known producers of active molecules, suggesting the possibility of selecting relevant extracts using this strategy.

Microbes belonging to the genus *Verrucosispora* possess significant chemical diversity and biological properties. They have attracted the interests of many researchers and are becoming promising resources in the marine natural product research field. A bioassay-guided isolation from the crude extract of *Verrucosispora* sp. strain MS100047, isolated from sediments collected from the South China Sea, has led to the identification of a new salicylic derivative, glycerol 1-hydroxy-2,5-dimethyl benzoate , along with three known compounds, Brevianamide F, Abyssomicin B, and Proximicin B. Compound 1 showed selective activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with a minimum inhibitory concentration (MIC) value of 12.5 μg/mL. Brevianamide F, which was isolated from actinobacteria for the first time, showed a good anti-BCG activity with a MIC value of 12.5 μg/mL that has not been reported previously in literatures. Proximicin B showed significant anti-MRSA (MIC = 3.125 μg/mL), anti-BCG (MIC = 6.25 μg/mL), and anti-tuberculosis (TB) (MIC = 25 μg/mL) activities. This is the first report on the anti-tubercular activities of proximicins.

In addition, *Verrucosispora* sp. strain MS100047 was found to harbor 18 putative secondary metabolite gene clusters based on genomic sequence analysis. These include the biosynthetic loci encoding polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) consistent with abyssomicins and proximicins, respectively. The biosynthetic pathways of these isolated compounds have been proposed. These results indicate that MS100047 possesses a great potential as a source of active secondary metabolites [70].

Cyclomarin A, a novel anti-inflammatory cyclic peptide, is one of three cyclomarin compounds isolated from marine *Streptomyces* spp. CNB-982. Recently, cyclomarin A was revived as an anti-TB lead compound based on results of whole cell-based screening assays. Cyclomarin A shows antibacterial activity against replicating M. tb in culture broth and in human-derived macrophages, with minimal inhibitory concentration (MIC) values of 0.3 and 2.5 μM, respectively. Exposure to 2.5 μM cyclomarin A for 5 days resulted in 90 % kill in non-replicating M. tuberculosis. Cyclomarin A is effective against MDR *M. tuberculosis*, contrary to the resistance shown by *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*. This activity of cyclomarin A suggests

that its cytotoxicity against *M. tuberculosis* is mediated by a novel mode of action, which distinguishes it from the existing anti-TB drugs. To identify target molecules of cyclomarin A, *M. tuberculosis* lysates was incubated with sepharose beads coupled to cyclomarin A and screened the bound proteins, which resulted in the identification of ClpC1 by proteomic analysis. In the mixture containing cyclomarin A and *M. tuberculosis* lysates, cyclomarin A was specifically bound to ClpC1 and this was confirmed by competition assays [71].

Lassomycin, an anti TB lead molecule was isolated from the rare actinobacteria Lentzeakentuckyensis spp. IO0009804. Lassomycin, refasan anti-TB lead compound, shows anti-TB activity against MDR and XDR M. tb, as well as to the drug-sensitive strain. The MIC ranges from 0.41 to 1.65 μM, and it is effective in killing inactive *M. tuberculosis* as well as exponential M. tb, unlike rifampicin, which is ineffective against inactive *M. tuberculosis* [72].

Ecumicin is a macrocyclic peptide produced by another rare actinobacteria, *Nonomuraea* spp. MJM5123. It is composed of 13 amino acids, including natural and highly methoxylated unnatural amino acids. Ecumicin shows promising anti-TB activity against MDR and XDR M. tb as well as the sensitive M. tb strain, with MIC values ranging from 0.16 to 0.62 μM. It effectively kills inactive M. tb with a minimal bactericidal concentration of 1.5 μM, indicating the possibility of shortening the duration of treatment [73].

Chrysomycin A from an actinomycete isolated from a coastal area in Kerala was purified by bioassay guided fractionation against *M. tuberculosis*. The authors reported that, for the first time, the chromomycin A has antimycobacterial activity. It was found to be bactericidal to planktonic and intracellular *M. tuberculosis* with an MIC of 3.125 μg/ml; it is non-hemolytic and has negligible cytotoxicity. The actinomycete that produces chrysomycin A was found to be a *Streptomyces* sp. through 16S rRNA gene sequencing [74].

2. Conclusion

The huge burden of tuberculosis and its associated plethora of challenges, makes it a herculean task to discover novel drugs against it. The knowledge of the problems of resistance and latency along with HIV co-infection leads to path of less explored avenues to be experimented to discover new metabolites with novel mechanism of action. Actinobacteria comes as a trusted savior for rescue as the success rate of tapping this goldmine has proved and evidenced as potential to alter the TB regimen.Thus experimental validation of the *invitro* results in animal models will pave way for preclinical studies to hasten the TB drug discovery facilitating the arrival of new drugs sooner.

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Chapter 3

Amylase: A Magnificent Enzyme Tool

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1. Introduction

Since past couple of decades, enzymes have been consistently used as an efficient industrial tool to synthesize a variety of products. Industries are highly dependent upon some basic enzymes viz Amylase, Carboxymethyl cellulase and proteases. Such pioneering enzymes find their applications in a wide range of industries, including pharmaceuticals, food and beverages industry and textile industry. Amylase were initially reported from the buds of tuber by Bailey [1].

1.1. Sources of enzymes

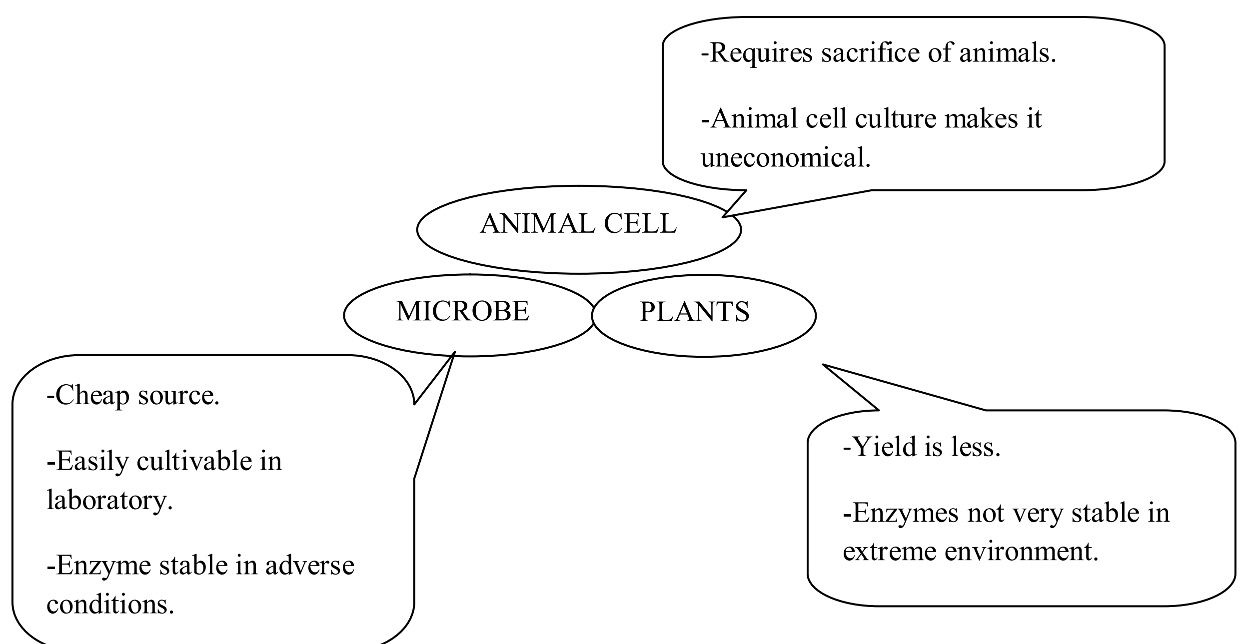

Figure 1: Sources of Amylases

In nature amylases are derived from three main sources:

(i) Animals
(ii) Plants
(ii) Microbial sources

Microbial sources of enzymes are more preferred over plants and animals because of their stability and high productive yield.

1.2. Amylases: An ancient enzyme

Amylase is well known enzyme and its potential industrial application gained recognition since 1970s. All amylases act upon the α–1,4–glycosidic bonds. As a class of diastase, Amylase is the first enzyme to be discovered and isolated in 1833 by Anselme Payen. Amylase initiates the chemical process of digestion of starchy food in mouth as it is present in saliva of humans and some animals. Plant cells also contain Amylase. The basic principle behind Amylase action is conversion of starch into sugars. Amylases are classified into three types-

(i) α–Amylase (EC3.2.1.1) also known as 1,4-α-D-glucan glucanohydrolase, glycogenaseis a calcium dependent metallo-enzyme and unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. Because it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase. In animals, it is a major digestive enzyme, and its optimum pH is 6.7–7.0. In human physiology, both the salivary and pancreatic amylases are α-amylases. The α-amylases form is also found in plants, fungi (ascomycetes and basidiomycetes) and bacteria (Bacillus).

(ii) β–Amylase (EC3.2.1.2) also known as 1,4-α-D-glucan maltohydrolase, glycogenase, saccharogen amylase) is also synthesized by bacteria, fungi, and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1,4glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit. The optimum pH for β-amylase is 4.0–5.0.

(iii) γ–Amylase (EC3.2.1.3) also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase cleaves α-1,6 glycosidic linkages, as well as the last α-1,4glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose. The γ-amylase has most acidic optimum pH of all amylases because it is most active around pH 3.

2. Recent Advances in Enzyme Technology

With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Considerable efforts have been devoted to the selection of microorganisms via sophisticated screening techniques and process methodology for the production of enzymes with new physiological/physical properties and tolerance to extreme conditions used in the industrial processes (e.g. temperature, salts and pH). The marine environment has proven to be a rich source of both biological and chemical diversity [3]. The optimum activity of marine bacterial enzymes usually occurs at high salinity, making these enzymes utilizable in many harsh industrial processes, where the concentrated salt solutions used would otherwise inhibit many enzymatic transformations. In addition, most marine bacterial enzymes are considerably thermo tolerant, remaining stable at room temperature over long periods.

3. Current Research on Amylase Catalyzed Starch Hydrolysis

Starch processing industries mainly derive starch from major crops like potato, rice and wheat. Conventionally, acid hydrolysis method is used for its degradation. Starch processing industries which are springing fast in past decades witnessed a paradigm shift from chemical hydrolysis to enzymatic hydrolysis for production of maltodextrins,modified starches, glucose or fructose syrups.

According to Marrel, 30% of the enzyme production in the world is focused on starch converting enzymes. The total starch converting enzymes essentially belonging to the amylase are reported to be 13 glycosyl hydrolases [4]. The studies on these thirteen enzymes by Crystallization and X- ray crystallography revealed a common structural architecture having 8 barrel structure protein.

Marine bacteria, corals and soft algae are reported to be novel sources of Amylase. The investigation revealed eight marine sedentary organisms, six marine bacteria, one soft coral and one algal isolates. All were reported to produce industrially important enzymes [5].

4. Future Prospects in Organic Matter Recycling in Marine Environment

Novel bacterial isolates were reported to produce either amylase or α-Carboxy methyl cellulaseor protease. Alcaligenes and Bacillus were reported to show highest activities amongst the isolates. These enzymes may find their commercial applications in catalyzed organic matter recycling. Hot springs were investigated for isolation of bacterial strains by Asif and Rasool [6]. It revealed Bacillus WA 21 had maximal yield at 55 ºC and pH 6.

5. Economical Approach Towards Enzyme Production

Since amylases and associated enzymes have great demand in sectors of pharmaceuticals, leather, laundry, food and waste processing industries, effectiveness of their production is very much targeted.

Agro industrial waste is the upcoming and widely accepted alternative due to its easy availability, diversity, high nutritive value and extremely low cost. Many reports of usage of Baggasse, molasses, cassava waste water, tea, coffee leaves, banana leaves, rice husk and paddy straw for amylase production have been recorded [7].

6. Experimental Designing for Optimization of Enzyme Yield

Designing of experiments was a newer approach towards optimization of enzyme production. Designing helps to evaluate the effect of interactions of different physicochemical parameters on enzyme production. Two approaches were taken by researchers for optimization: viz Smf and SSF. Negi and Banergee employed Evolutionary Operation (EVOP) factorial design technique in amylase production [8]. Experimental runs were carried out in a bioreactor by modified solid state fermentation. The EVOP technique was applied on indigenously isolated strain *Aspergillusawamori* MTCC 6652 . The result supported amylase production upto levels of 9420.6 IU/g at 37ºC, pH 4.00 and relative humidity 85%.

Smf approaches were also taken up by various scientists [9,10]. Experiments on influence of various organic and inorganic nitrogen sources are carried under optimization. Ammonium nitrate (450/ mg), maltextracttryptone are some of the preferred Nitrogen sources supporting amylase production. Optimization of thermal stability of the enzyme was also carried out. The thermal denaturation studies in presence of trehalose, sorbitol, sucrose and glycerol were carried out due to their industrial relevance [11]. The denaturation stabilities are aided by techno based intrinsic and 8–anilinonaphathalene 1–sulphonic acid fluorescence studies revealing exposure of hydrophobic cluster on protein surface.

SSF was reported to be superior during Amylase production experiments based on wheat bran and rice bran as substrates for enzyme production. Oyeleke et al tried to utilize Africa lowest beans as a substrate for amylase production coupled with protease [12]. Optimization of pH and temperature revealed highest yield of 0.87 mg / 1ml/sec. Enzyme characterization experiments revealed Km and Vmax values. Influence of activators and inhibitors studies helped to explore the Kinetic behavior of the enzyme.

7. Optimization of Amylase Production

The general optimal parameters investigated by various researches were pH of medium, incubation temperature, influence of various carbon and Nitrogen sources on enzyme produc-

tion. Different sources of enzymes have exhibited different optimal parameters with respect to pH, temperature, carbon sources and nitrogen sources as given in the following **Table**.-

Table 1: Different microorganism and optimization parameter for the production of α-Amylase

Bacteria strain		
Organism	**Optimization parameter**	**Authors**
B. amyloliquefaciens (MTCC 1270)	calcium (Ca^{+2}), Nitrate (NO_3^-), and chloride ions (Cl^-)	Saha et al., 2014 [13]
Bacillus amyloliquefaciens	Wheat bran	Abd-Elhalem et al., 2015 [14]
Bacillus amyloliquefaciens	37ºC, pH =7	Francis et al., 2003 [15]
Bacillus cereus	Cow dung	Vijayaraghavan et al., 2015 [16]
Bacillus cereus	35ºC	Kumari et al, 2017[17]
Bacillus cereus KR9	Starch, Peptone, pH = 10	Krishma and Radhathirumalaiarasu, 2017 [18]
B. licheniformis NH1	75ºC, pH = 6.5	Haqet al., 2002 [19]
Bacillus licheniformis	Wheat starch	Hmidetet al., 2010 [20]
Bacillus licheniformis	70ºC, pH = 6.5	Bozicet al., 2011 [21]
Bacillus licheniformis	60ºC, pH = 7	Muralikrishna and Nirmala, 2005 [22]
Bacillus sp. A3-15	65ºC, pH = 8.5	Arikan, 2008 [23]
Bacillus sp. ANT-6	37ºC, pH = 7	De-Souza and Martins, 2000 [24]
Bacillus sp. BBXS-2	Lignocellulosic biomass	Qureshi et al.,2016 [25]
Bacillus sp. IMD 434	Peptone	Kanwal et al., 2004 [26]
Bacillus sp. IMD 434	Yeast extract	Natasha et al., 2011 [27]
Bacillus strain	50°C and pH 8.	Simair, 2017 [28]
Bacillus subtili *Bacillus licheniformis*	Rice starch	Sarojaet al., 2000 [29]
Bacillus subtilis	Arginine	Haq et al., 2010 [30]
Bacillus subtilis	Corn starch	Lene et al., 2000 [31]
Bacillus subtilis *Bacillus licheniformis*	Potato starch	Bilal and Figen, 2007 [32]
Bacillus subtilis	50ºC, pH = 7	Shiau and Hung, 2003 [33]
Bacillus subtilis	50°C	Hassan and Khairiah, 2014 [34]
Bacillus subtilus KCC103	Cane sugar	Patel et al., 2005 [35]
Bacillus MJK1, MJK2, MJK6 and MJK10	pH = 8.0, 50°C and 72 hrs	Kanimozhi et al, 2015 [36]

Yeast strain		
Saccharomyces cerevesiae	pH = 6.0, initial temperature 20ºC	Jayalakshmi and Umamaheswari, 2015 [37]

Mold strain		
Aspergillusflavus	Ammonium nitrate	Sajitha et al., 2010 [38]
Aspergillusflavus;		
Aspergillusoryzae	Maize starch	Niaziet al., 2010 [39]
Aspergillusniger	pH = 6.0, initial temperature 30ºC	Ire et al., 2017 [40]
Aspergillusniger	30ºC, pH = 5.5	Afifi et al., 2008 [41]
Aspergillusniger BTM-26	Wheat bran	Abdullah et al., 2014 [42]
Aspegillusniger, *Corynebacterium gigantean*	Ammonium sulphate Casein	Riaz et al., 2007 [43]

8. Applications of Amylases

The major industry which requires starch hydrolysis with various product implications is food industry. The differential hydrolysis of starch can be facilitated through novel enzyme source combined with variable hydrolytic conditions. The main products resulted through the amylases are glucose syrups, fructose syrups, clarified beverages and fruit juices, dextrose powder, dextrose crystals, malto-oligosaccharides, maltosyrups, and Alo- mixtures.

Amylases are also used for imparting sweetening to the dough, acceleration rates of fermentation in bakery products, improving palatability of bakery items. Other than food and bakery industry, amylases are utilized in detergents, direct ethanol production and textile industry.

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Chapter 4

Endophytic Fungi, A Versatile Organism for Modern Microbiological Research and Industrial Applications

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Abstract

Endophytic fungi are one of the least studied plant microbe- interactions. In this chapter ecology, taxonomy, bioactive compounds and enzyme production by endophytic fungi were discussed. Finally a general procedure for isolation of endophytic fungi from plant parts is given.

1. Introduction

The word endophyte was primarily coined by De Bary in 1866 (Wilson, 1995). Ever since then it has undergone many subtle changes. Suryanarayanan [2] reported that the horizontally transmitted endophytic fungi establish endosymbiotic relationship with host plants of all lineages. Endophytic fungi occur universally and they have been reported in plants from different ecological niches. Further, these groups of fungi have the ability to produce an array of secondary metabolites showing different bioactivities and resulted in the use of these fungi for biotechnological applications. David *et al*., [3] quantified the associations between fungal endophytes and biocontrol-induced herbivory of invasive purple loosestrife (L.). *Lythrum salicaria* and Suryanarayanan *et al*., [4] studied the role of endophytic fungi as biocontrol agents. Recently, Raman and Suryanarayanan, [5] described that fungus plant interaction influences plant-feeding insects behavior. The endophytic fungi are also involved in multiple balanced

antagonisms and it was revealed by Schulz et al. [6]. Szink, et al., [7] showed a new evidence for broad trophic status of leaf endophytic fungi of *Quercus gambelii*. The chemical interactions of endophytic fungi can be exploited as microbial factories for sustainable applications in biotechnology [8].

The term endophyte itself has evolved to accommodate our growing knowledge on these fungi. Ainsworth [9] defined an endophyte as “a plant living inside another organism”. Carroll [10] restricted its usage to organisms that cause asymptomatic infections within plant tissues; this description excluded pathogenic fungi. It was further widened by Petrini [11] with an explanation to include all organisms inhabiting plant organs at some time in their life. He further stated that they can colonize internal plant tissues without any obvious damage to the host. This definition would include latent pathogens and epiphytes which are established as endophytes at some stages of their life. Thus, some endophytes residing in plant tissues become pathogenic when their hosts are weakened [12]. Wilson [1] proposed that endophytes are fungi or bacteria which, for their entire or part of life cycle, occupy the living plant tissue. They do not cause any apparent and symptomatic infections within plant tissues but only symptoms of disease.

Sampson [13] reported the occurrence of endophytic fungi in plant tissues such as *Lolium* grass. The presence of endophytes in *Pseudotsuga menziesii* was reported by Bernstein and Carroll [14] and it was considered as a contemporary renaissance of research on endophytic fungi. Apart from diversity of endophytic fungi, the study on the metabolites produced by them offers a diverse knowledge of their pharmaceutical, medical and agricultural importance.

2. Taxonomy of Endophytic Fungi

The endophytic fungi present in plant parts above the ground generally belong to the Ascomycetes, Coelomycetes and their conidial (anamorphic) forms and Hyphomycetes forms lacking a sexual state. Petrini [15] reported that rarely a small amount of Basidiomycetes and Oomycetes occur as endophytes. He also classified the endophytic fungi assemblage of a plant into two broad categories – the non-specific, epiphytic and the true endophytic. Every effort to isolate endophytic fungi from a plant is certain to yield quite a few forms and prevent their explicit classification. Therefore, mycologists take recourse to the culture characteristics of these fungi to make a distinction from one another [16,17].

3. Ecology of Endophytic Fungi

Endophytic fungi are omnipresent. They have been isolated from varied groups of plants such as marine algae [18], mosses and ferns [15] and coniferous trees [19,20]. Some angiosperms such as members of the Poaceae [21,22,23] and Ericaceae [15] have been studied for

the incidence of endophytic fungi in them. Investigation on species composition of endophytic fungal communities of many hosts has shown that a large number of endophytic fungal taxa could be isolated from a lone host species [24]. In general, one or a few endophytic fungal species dominate in a host plant [11,17].

The environmental factors and age of the host tissue are known to influence the distribution of endophytic fungi in a host. The quantity of endophytic fungi obtained from the host tissue decreases with the age [25,26]. Endophytic fungi samples collected simultaneously from *Pinus sp* and *Fagus sp* growing at similar environment and site showed that the endophyte assemblages of these two plants are dissimilar [27]. This shows that endophytic fungi that colonize trees are known to be host specific. Similarly, the endophyte community of a plant is also affected by the quality of air [11]. All these studies show that the endophytic fungi occupy a unique niche. Despite the fact that they may not be exposed to the vagaries of the environment as the Phylloplane fungi are, they have to come across the defense reactions of the host. Hence, their living strategies are likely to be unusual from those of other fungi.

4. Biotechnological Potential of Endophytic Fungi

Endophytic fungi inhabit a very unique and often hostile habitat. Recently they are recognized as sources of new metabolites useful in biotechnology and agriculture [28]. Numerous endophytic fungi cultures produce antibiotic compounds that are active against human and plant pathogens. Fisher *et al.*, [29] showed antifungal and antibacterial activity in more than 30% of the endophytic fungi that they have tested. A broad spectrum antibiotic was isolated by Fisher *et al.*, [30] from an endophyte of *Vaccinium sp*. Seed germination in ivy was influenced by endophytes such as *Aureobasidium pullulans* and *Epicoccum purpurascens* by producing phytohormones [31]. In developed countries, endophytic fungi are viewed as biocontrol agents for plant diseases [32,11,33]. They are always the preferred components of the fungus-plant system and are ideal for experimental manipulations. Research shows that valuable attributes could be introduced into host plants for desirable qualities from endophytic fungi representing natural genomes. Endophytic fungi could also be used as gene vectors and artificially introduced into a population of host plants [31].

Endophytic fungi readily integrate into host systems and hence their associations offer great possibilities for the biocontrol programme [32,33]. Dewan and Sivasithamparam [34] isolated an endophytic fungus from wheat and reported a significant defense against infections by “Take all’ fungus in host plant. Endophytic fungi present in conifer needles produce chemicals that can ward off insect pests. The endophyte-mediated antagonism towards an insect pest in Douglas fir is an excellent example. Their needles harbor an endophytic fungus, *Rhabdocline parkeri* that controls the gall midge, *Contarina* sp [19]. These studies show that endophytic fungi are prospective candidates for biocontrol programme [35,36].

Pharmaceutical and agricultural industries have equally explored the Endophytic fungi [37] representing an untapped pool of secondary metabolites [38,76]. In INBIO project with the collaboration of Merck Research Laboratories, USA and Costa Rica, endophytic fungi from forest trees are screened for novel antibiotics [39,40]. Recently, Li *et al*. [41] reported Taxol, an anticancer drug produced by endophytic fungi isolated from the bark of *Taxus wallachiana* growing in Himalayas of Nepal.

5. Endophytic fungi as a resource of Bioactive Metabolites

This write-up reveals the significance of endophytic fungi from plants as a source of bioactive metabolites. Endophytic fungi produce various metabolites belonging to diverse structural groups like terpenoids, steroids, quinines, phenols, coumarins etc. [42]. They can also be termed as chemical producers inside plants [43,44] . Tejesvi *et al*., (2007) reported that endophytic fungi of medicinal plants produce secondary metabolites which can be studied for curing several diseases. All these studies reveal that Endophytic fungi represent a chemical reservoir of novel compounds such as which offer antimicrobial, antiviral, antifungal, anticancer, antiparasitic, antitubercular, antioxidant, immunomodulatory, insecticidal and many uses in pharmaceutical and agrochemical industries [42].

Fungal endophytes serve as a storehouse for various novel compounds with immense value in agriculture, medicine and various other industries [45]. Owing to their constant interaction with host plants, the secondary metabolites or the bioactive compounds produced by endophytic fungi are unique and distinct compared to those produced by soil fungi or fungi associated with algae [46,47]. Endophyte - plant interactions are different from pathogen - plant interactions. In Endophyte - plant interactions, neither the host nor the fungus gets detrimental effects [48,49,50]. This is beneficial to bioprospectors as the endophytes confer selection pressure to develop novel metabolic pathways due to their sustained and prolonged reactions against the defense mechanism of the host [51,52,53,54,55].

A well-known anticancer drug paclitaxel was initially produced from the yew (*Taxus brevifolia*) species. Paclitaxel prevents polymerization of tubulin molecules during the process of cell division and is helpful in treating a number of tissue proliferating diseases in human [56,57]. The usefulness of paclitaxel led to the extensive research on endophyte producing the compound which was identified as Taxomyces andreanae from the plant *T. brevifolia* [58]. Anticancer activity of the endophytic fungi is based on the bioactive compounds present in it. The anticancer potential of endophytic fungi has been studied extensively between 1990 and 2013 in which the endophytes *Pestalotiopsis* and *Aspergillus* have been explored for their anticancer compounds [59].

Suryanarayanan *et al.*, [60] screened the anti-cancer activity of 110 isolates of endophytic fungi in mouse fibroblast cell line L-929. Using the organic solvent extracts of the

endophytes isolated, the cytotoxic effects were assayed using MTT colourimetric assay. Endophytic microbial products act as potent antimicrobial agents against various human pathogens. The endophyte Pestalotiopsis neglecta was explored for its antimicrobial property against the human pathogens. The phenolic compound tyrosol produced by the endophyte *Diaporthe helianthi* isolated from the plant *Luehea divaricata* was studied for its antagonistic effects on pathogenic strains of bacteria [61]. Singh and Kaur [62] isolated thirty-six endophytic fungi from *Acacia nilotica* and screened them for the production of alpha amylase and glycosidase inhibitors. The endophyte *Aspergillus awamori* isolated from *Acacia nilotica* possesses antidiabetic property. Over 75% of the antibacterial drugs in clinical use are of natural origin and most of them are obtained from fungal sources. Antioxidant activity was observed in the compounds pestacin and isopestacin produced by endophyte *Pestalotiopsis microspora* isolated from *Terminalia morobensis*. The antioxidant activity was due to the structural similarity of isopestacin with flavonoids [63].

In fungi, the genes coding the enzymes required for secondary metabolic pathways are present as gene clusters situated in the same locus [64,65]. These gene clusters are co-expressed and evolve rapidly through horizontal gene transfer and multiple rearrangements. These are conducive for novel chemical synthesis by fungi [66]. It is necessary to screen fungi under different culture conditions for its novel bioactive production. Culture conditions such as growth media composition, pH, temperature, incubation time and the addition of various growth regulators can influence the secondary metabolite profile of the endophytic fungi and also aid in the synthesis of new products [67]. For instance, Squalestatin S1, produced by a Phoma sp. acts as an inhibitor of squalene synthase which is essential for the biosynthesis of cholesterol. Several fungal species produce squalestatin and it is produced in higher quantities in Phoma sp. Parra *et al.*, [68] optimized the composition of fermentation medium for increased squalestatin production.

6. Anticancer and Antioxidant Compounds

Endophytic fungi have been considered as a source of anticancer compounds since the million dollar drug discovery Taxol from the endophytic fungus Taxomyces andreanae from the bark of *Taxus brevifolia* [58]. In the current chapter only few examples of anticancer compounds have been given in (**Table-1**). Antioxidants defend the cells from the harm induced by unstable free radicals in the cells. The Free radicals cause or induce diseases like cancer and degenerative diseases like Alzheimer's etc. However the fact is, only few antioxidant compounds are reported and approved. The need of the hour is to search for new, effective and eco-friendly antioxidants and endophytic fungi are one of the most promising sources. Few antioxidant compounds reported in Table 1.

7. Immunomodulatory Compounds

Immunomodulatory compounds are produced by several microbes. In recent years, fungi are viewed as promising producers. Immunomodulatory compounds are either immunosuppressive or immunoregulatory drugs [42]. There is a huge demand for immunosuppressive compounds and endophytic fungi are the producers of several immunosuppressive compounds. Some of the major immunosuppressive compounds listed in Table 2.

8. Endophytic Fungi as Source of Industrial Enzymes

The production of extracellular enzymes such as cellulases, pectinases, esterases, amylases, proteases, tyrosinases, tanneases, chitinases, L-asparaginase by few endophytic fungi has been studied by substrate utilization test and isozyme analysis [69]. The production of growth hormone Indole Acetic Acid (IAA) has been demonstrated in endophytic fungi such as *Aureobasidium pullulans* and *Epicoccum purpurascens* [70]. A Sterile endophytic fungi and *Fusarium* sp isolated from *Azadirachta indica* produced IAA in culture [33]. Carroll and Petrini [71] investigated the capability of endophytic fungi to tolerate or metabolize phenolics and other defense chemicals of the host tissues. Some endophytic fungi are known to produce compounds that interfere with plant cell division [17].

9. In Silico Studies on Endophytic Fungal Secondary Metabolites

Using modern computational methods, the bio-macromolecule can be designed to bind with the identified target to change a particular biochemical process. When a three dimensional structure of a protein is known, it becomes easy to identify a compound that binds with its active site using modeling techniques [72,73]. Several compounds of natural origin have excellent therapeutic uses against various pathogens. Screening a large number of compounds is a complicated step in research. This is possible by virtual screening of a large number of compounds using modern computational tools and molecular data banks [74]. In silico studies on endophytic fungal secondary metabolites is less explored. Kandasamy *et al*., [75] reported the in silico study on metabolites from *Trichoderma* sp. against 4,5-Diarylisoxazole HSP90 Chaperone, a skin cancer protein. The compounds from *Trichoderma* sp. were also tested for their drug likeness by Lipinski's rule.

Table 1: List of some of the anticancer and antioxidant compounds produced by endophytic fungi from various hosts

S.No.	Endophytic Fungus	Host Plant	Compound
1.	*Taxomyces andreanae*	*Taxus brevifolia*	Taxol
2.	*Pestalotiopsis microsporum*	*Torreya taxifolia*	Torreyanic acid
3.	*Entrophospora infrequens*	*Nothapodytes foetida*	Camptothecin
4.	*Fusarium solani*	*Camptotheca acuminata*	Topotecan

5.	*Aspergillus fumigates* *Philalocephala fortinii* *Fusarium oxysporum*	*Juniperus communis* *Podophyllum peltatum* *Juniperus recurva*	Podophyllotoxin
6.	*Leaf Endophyte*	*Mimusops elengi*	Ergoflavin
7.	*Mangrove Endophyte*	*Mangroves*	Secalonic acid D
8.	*Rhinocladiella sp*	*Tripterygium wilfordii*	Cytochalasin Cytochalasin H Cytochalasin J Epoxycytochalasin
9.	*Chaetomium globosum*	*Imperata cylindrica*	Chaetoglobosin
10.	*Mycelia Sterilia*	*Catharanthus roseus*	Vincristine
11.	*Fusarium oxysporum*	*Annona squamosa*	Polyketide
12.	*Alternaria sp*	*Taxus cuspidata*	Paclitaxel
Antioxidant Compounds			
13.	*Pestalotiopsis microspora*	*Terminalia morobensis*	Pestacin Isopestacin
14.	*Cephalosporium sp.*	*Sinarundinaria nitida*	Isobenzofuranone
15.		*Trachelospermum jasmi-noides*	Graphislactone A
16.	*Fusarium sp.*	*Cajanus cajan*	Cajaninstilbene acid
17.	*Xylaria sp.*	*Ginkgo biloba*	Phenolics
18.			Flavonoids
19.	*Chaetomium sp.*	*Nerium oleander*	Flavonoids
			Phenolic acids

Table 2: List of major Immunosuppressive compounds produced by endophytic fungi from various hosts

S.No.	**Endophytic Fungus**	**Host Plant**	**Compound**
1.	*Cytonaema sp.*	*Medicinal Plant*	Cytonic acid A & B
2.	*Unidentified endophyte*	*Quercus coccifera*	Torreyanic acid
3.	*Pullularia sp*	*Unidentified tree*	Pullularins A-D
4.	*Pestalotiopsis theae*	*Unidentified tree*	Pestalotheol-C
5.	*Aspergillus fumigates* *Philalocephala fortinii* *Fusarium oxysporum*	*Juniperus communis* *Podophyllum peltatum* *Juniperus recurva*	Podophyllotoxin
6.	*Leaf Endophyte*	*Mimusops elengi*	Ergoflavin

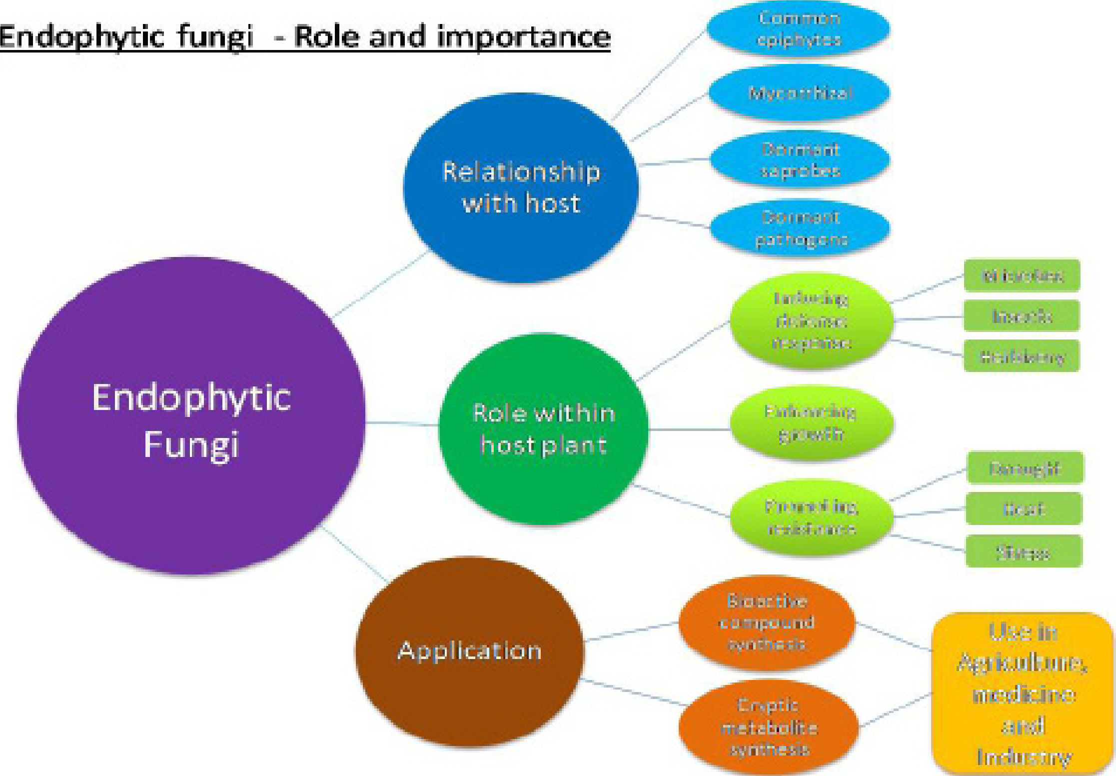
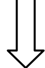


Figure 1: General procedure for the isolation of Endophytic Fungi from various tissues of plant

Sterilization of various plant segments

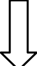
Collect undamaged and healthy plant tissues-Leaf/Petiole/Stem/Bark/Seed



Wash thoroughly in running tap water

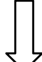
Cut Leaf/Petiole/Stem/Bark/Seed into 5-8 mm segments

Surface-sterilize the segments using in 70% ethanol for 30 seconds and immerse in 4% Sodium hypochlorite for 30 seconds and rinse in autoclaved double distilled water

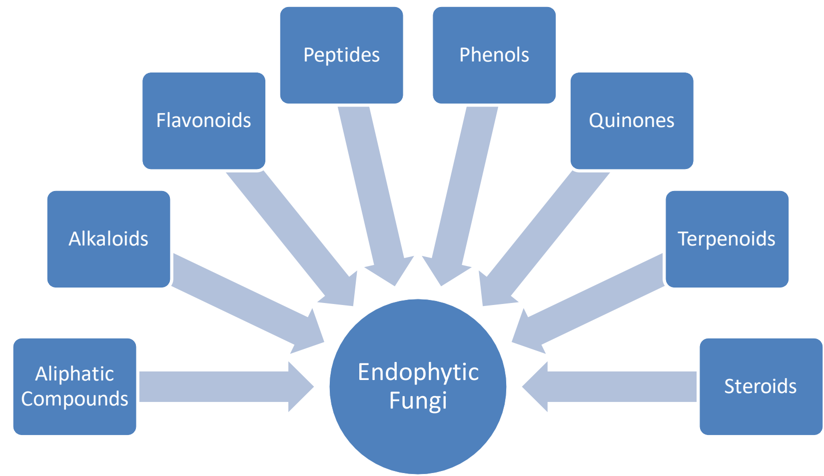


Place the segments (4-6) in Petri Plate containing suitable media with a antibiotic

Incubate the inoculated plates in light chamber for 3-4 weeks for the growth of endophytic fungi



Identify the endophytic fungi by Conventional (Morphological/Conidial/Fruit body structures) and Molecular Method (ITS primers)



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Chapter 5

Cholesterol Oxidases: Microbial Enzymes in Industrial Applications

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Abstract

Cholesterol oxidases are bifunctional flavoenzymes produced by diverse bacterial species. These enzymes catalyse the oxidation of steroid substrates containing a hydroxyl group at the 3β-position of the steroid ring backbone. The enzyme exists in two main forms: one with the FAD cofactor embedded noncovalently in the enzyme; and one with the cofactor attached covalently to the protein. Cholesterol oxidase is of significant importance owing to its use in analysis of cholesterol amount in various clinical and food samples. In addition, the enzyme also acts as a larvicide biocontol agent against many insects and is also involved in the biotransformation of a number of steroids. Moreover, cholesterol oxidase is also associated with some bacterial infections and thus can be explored as a potential new antimicrobial drug target to contain bacterial infections.

1. Introduction

The history of cholesterol oxidase dates back to over 70 years when search for cholesterol-degrading microorganisms was started and many species of *Mycobacterium* were observed to use cholesterol as a source of carbon and energy [1,2]. In eukaryotes, cholesterol is essential for maintaining cell membrane structure and for synthesizing a number of important compounds. Cholesterol is either derived exogenously or synthesized endogenously in the endoplasmic reticulum [3]. An abnormally high level of cholesterol in the blood leads to heart disease, in addition to many other severe health problems. Higher cholesterol levels (greater than 200 mg dL^{-1}) may cause hypertension, arteriosclerosis, lipid metabolism dysfunction, brain vessel thrombosis, nephritis, diabetes, jaundice and cancer. Alternatively, abnormally low cholesterol levels may cause hyperthyroidism, anemia and malabsorption [4,5]. Several

compounds such as statins, filipin and cyclodextrins may be used to manipulate cellular cholesterol [6]. Cholesterol oxidase is a bacterial enzyme that has proven to be very useful in biotechnological applications related to the detection of cholesterol and to the disruption of cholesterol-containing membranes. Cholesterol oxidase is commonly used to manipulate cholesterol levels of cells [7].

Cholesterol oxidase has many applications and one of most important one is its wide use for the detection of amount of cholesterol present in food, serum and other clinical samples through an enzymatic assay [8,9]. With the exception of glucose oxidase, this enzyme has become one of the most widely used biosensors in clinical applications [10]. Cholesterol oxidase is also used for the production of a variety of intermediates which are precursors for the synthesis of steroid hormones, such as 4-androstene-3, 17-dione (AD) and 1, 4-androstadiene-3,17-dione (ADD) [11]. Cholesterol oxidase plays a critical role in sterol catabolism by converting 3-β-hydroxysteroids to 3-oxo-4-enesteroids [10,12,13]. Cholestenone formed by catalysis of cholesterol by cholesterol oxidase is also an important synthetic intermediate in many steroid transformations. Previous studies have shown that it is effective against obesity, liver disease and keratinization [14]. Additionally, the enzyme has insecticidal effect against larvae of some insects [15], also used for membrane structure analysis and hypothesised to act as a signalling molecule for the biosynthesis of polyene macrolide pimaricin.

Cholesterol oxidase (3-β hydroxysterol oxidase, EC 1.1.3.6) is a member of flavin adenine dinucleotide (FAD) dependent enzymes super family and catalyzes the oxidation of cholesterol (cholest-5-en-3β-ol) to its 3-keto-4-ene derivative, cholestenone (cholest-4-en-3-one), with the reduction of oxygen to hydrogen peroxide [16]. However, some bacterial cholesterol oxidases have also been reported to catalyse oxidation of cholesterol to 6β-hydroperoxycholest-4-en-3-one (HCEO) in place of cholest-4-en-3-one (CEO) [18]. Cholesterol oxidase is a bacterial FAD-dependent enzyme which is found to exist in two different forms on the basis of bonding between enzyme and FAD cofactor. In first form the enzyme and its FAD cofactor are linked non-covalently (class I) and in second form cofactor is bound covalently to the enzyme (class II) [18,19]. Cholesterol oxidase is among the well-studied enzymes with prominent characteristics, structural features [20], broader biotechnological applications [21] and physiological functions [22,23]. Nonetheless, reports regarding cholesterol oxidase production, purification, molecular characterization and genetic analyses have been previously published [24].

Many bacteria belonging to the genera *Brevibacterium* [25], *Streptomyces* [26,27], *Corynebacterium* [28], *Arthrobacter* [29], *Pseudomonas* [30], *Rhodococcus* [31,32], *Chromobacterium* [17] and *Bacillus* [33,34] are reported to degrade cholesterol. Microbial cholesterol break down occurs by the degradation of its side-chain and the ring to acetyl CoA and propionyl CoA through a multi-step process and the enzyme which catalyzes the first step is

cholesterol oxidase. Most of steroids including cholesterol do not seem to be able to diffuse to the bacterial cytoplasm, and appears to be transported by specific uptake systems before being metabolized. In most of cholesterol degrading microorganisms, the cholesterol oxidase is employed in the initial step of cholesterol metabolism, while in case of pathogenic bacteria it acts as membrane-damaging factor contributing as a virulence factor and adds to the pathogenicity of these bacteria [35,36].

Figure 1: The reaction mechanism of cholesterol oxidase.

2. Sources of Cholesterol Oxidase

Cholesterol oxidase has been isolated from various bacterial sources, comprising both Gram-negative and Gram-positive bacteria [10,23]. Cholesterol oxidase producing bacteria produces it in three forms: intracellular, extracellular and membrane bound. Most of the microorganisms described as cholesterol oxidase producers, produce it in extracellular form which is eventually released into the fermentation medium. The cell-surface-linked form of enzyme is extractible with non-ionic detergents such as Triton X-100 (polyethylene glycol octylphenyl ether) and Lubrol PX (polyethylene glycol monododecyl ether) [37,38,39,40].The most common microbial sources for production of cholesterol oxidase are Arthrobacter [41,42], *Bacillus* [33,43], *Brevibacterium* [44], *Bordetella* [45], *Corynebacterium* [28], *Mycobacterium* [46,47], ***Nocardia*** [48,49], *Rhodococcus* [32,38] and *Streptomyces species*. [26,50,51]. Some Gram-negative bacteria such as *Burkholderia* [52], *Chromobacterium* [17], *Enterobacter* [53] and *Pseudomonas species*. [30] have also been reported to produce cholesterol oxidase.

Cholesterol oxidase is a monomeric enzyme which exists in two forms classified as class I and class II, in class I the FAD cofactor is attached non-covalently to the protein and in class II this cofactor is bound covalently to the enzyme [54,55]. However these two classes of enzyme show same catalytic activity but do not have any significant sequence homology and thus appear to belong to different protein families [23]. Class I enzymes are part of the glucose-methanol-choline (GMC) oxido-reductase family and have been found mostly in actinomycetes such as *Streptomyces, Brevibcterium, Rhodococcus, Arthrobacte, Nocardia* and *Mycobacteriu*m spp. Whereas, Class II enzymes have been assigned to vanillyl alcohol oxi-

dase (VAO) family and have also been reported from *Brevibacterium sterolicum, Rhodococcus erythropolis* and some Gram-negative bacteria such as *Burkholderia* sp., *Chromobacterium* sp. and *Pseudomonas aeruginosa* [56]. Most of flavoenzymes contain a consensus sequence made up of repeating glycine residues (GXGXXG) continued by the presence of aspartic acid/ glutamic acid approximately 20 residues further is indicative of a nucleotide-binding fold [57]. The noncovalent form of cholesterol oxidase shows a nearly identical consensus sequence of glycine moieties (G17-X-G19-X-G21- G/A22) which is followed by a glutamate and confirms the presence of nucleotide-binding fold. However, the second form which exhibits covalent binding lacks this consensus sequence which indicates the probable absence of a nucleotide-binding fold [20]. Despite exhibiting structural differences both covalent and noncovalent enzyme forms possess a buried hydrophobic pocket for binding steroid ring backbone. Based on function, cholesterol oxidase contains two domains, the FAD-binding domain and the substrate-binding domain. There is no significant sequence identity between two types of enzymes resulting in different tertiary structure and kinetics mechanism [20,58]. It has been reported from different structural and kinetics studies that His447 and Glu361 residues act as main catalysts along with the conserved water molecule H_2O and Asn485 in type I cholesterol oxidase [59]. The 3-OH group of the steroid ring is linked to both the FAD cofactor and a bound water molecule *via* hydrogen bonding. The critical residues composing the active site of type II cholesterol oxidase from *Brevibacterium sterolicum* include Arg447, Glu475, Glu311, and Asn516 [54].

Cholesterol oxidase is thus a functional flavo enzyme which catalyzes the oxidation and isomerization of cholesterol to 4-cholesten-3-one in three successive steps (**Figure 1**). The first step results in the 3-OH group dehydrogenation with the loss of the 3α- hydroxy and 3β-hydroxy from the steroid ring backbone (reductive half-reaction). The two resulting electron equivalents are transferred to the oxidized FAD enzyme cofactor that is thence converted to its reduced form in the process. In the next step, the reduced form of FAD cofactor reacts with molecular oxygen (O_2) to regenerate original enzyme in its oxidized form and H_2O_2 (oxidative half-reaction). In the final third step, the cholesterol oxidase catalyzes isomerization of double bond in the steroid ring backbone, from Δ5-6 to Δ4-5, leading to final product formation. It has also been reported previously that cholesterol oxidase from *B. cepacia, Pseudomonas* spp., and *Chromobacterium* sp. oxidizes cholesterol to HCEO [17,60] The HCEO formation scheme differs only for a single step in which HCEO is formed from cholest-5-en-3-one, presumably by auto-peroxidation [23].

3. Properties of Cholesterol Oxidase

Cholesterol oxidases are among well studied enzymes and have been reported from several microorganisms. General range for molecular weight (Mr) of cholesterol oxidase is reported to be from 47–61 kDa. Cholesterol oxidases from different microbial sources are found

to be optimally active at neutral pH and are stable over a wide pH range. The enzymes have temperature optima in the range of 37–60°C (**Table 1**). Cholesterol oxidase from *Streptomyces fradiae* is reported to have highest optimum temperature of (70°C) among the enzymes reported [51]. Cholesterol oxidase produced from *Chromobacterium* sp. strain DS-1 is found highly thermostable retaining 80% of its original activity even at 85°C after 30 min [17]. Generally cholesterol oxidase does not require metal ions for its activity but in case of some enzymes, its activity was enhanced in the presence of metal ions [45,32]. Different chelating agents like EDTA, o-phenanthroline, and 8-hydroxyquinoline also do not seem to have any significant effect on the enzyme activity [17,61]. Since cholesterol is an insoluble compound, detergents as well as organic solvents are often added to the reaction solution to act as solubilizer(s). Cholesterol oxidase has been used for the optical resolution of non-steroidal compounds, allylic alcohols [62,63] and the bioconversion of a number of 3β-hydroxysteroids in the presence of organic solvents [64,65]. Therefore, an organic solvent-tolerant cholesterol oxidase would be useful for these applications. Organic solvents often influence the cholesterol oxidase activity [49,66].

Table 1: Properties of cholesterol oxidases produced from different microbial sources.

Producer organism	Optimum pH	Optimum Temp. (°C)	Mr (kDa)	Temp. stability (°C)	Km (mM)	Specific activity (Units/mg)	References
Brevibacterium sterolicum	6.5	55	46.5	-	0.03	55.2	[44]
Streptomyces aegyptia NEAE 102	7.0	37	46	50	0.152	16.1	[27]
Bacillus subtilis	7.5	37	105	37	3.25	1.39	[33]
Bordetella sp.	7.0	37	55	50	0.556	20.8	[45]
Chryseobacterium gleum	6.75	35	60	-	0.50	15.5	[67]
Rhodococcus sp.	8.0	37	60	-	-	35.6	[32]
Enterococcus hirae	7.8	40	60	92	-	124.9	
Streptomyces sp.	7.2	50	55	65	0.02	20.0	[26]
Chromobacterium sp. DS-1	7.0	65	58	85	0.026.	13.9	[17]
Burkholderia cepaca ST-200	6.8-8.0	60	60	70	-	16.9	[52]
Pseudomonas aeruginosa	7.0	70	59	70	92.6	11.6	[68]

4. Substrates for Cholesterol Oxidase

Cholesterol oxidase is active on a large number of sterols with or without a C-17 alkyl chain. However, it is most active in the presence of cholesterol. Richmond (1973) reported that cholesterol oxidase from a *Nocardia* sp. was affected largely by the length of side chain [48]. Long side chains appeared to aid the orientation of the substrate with respect to the active enzymatic site. Allain et al., (1974) found that ergosterol, 5,7-cholestadien-3β-ol, 20α-hydroxycholesterol, 5α-cholestan-3β-ol and 7-cholesten-3β-ol were oxidised at rates lower than that of the rate of cholesterol for the same enzyme [69]. In contrast to the enzyme from *Nocardia* sp., a cholesterol oxidase from *Brevibacterium sterolicum* was fairly reactive to substrates lacking a side chain [25]. The substrate specificity of cholesterol oxidase from *Streptomyces cinnamomeus* was studied [70] in oriented sterol monolayers and it was found that the cholesterol analogue 5α-cholestan-3β-ol was oxidized almost as fast as cholesterol itself. In addition, when the Δ-5 double bond in cholesterol was instead at the Δ-4 position and Δ-5 double bond was at the Δ-7 position (5α-cholest-7-en-3β-ol), the oxidation rate became slower. With C-17 side chain analogues of cholesterol, it was observed that the complete lack of the C-17 side chain (5-androsten-3β-ol) or the insertion of an unsaturation at Δ-24 (desmosterol) or even an ethyl group at C-24 had no appreciable effects on sterol oxidation rate, implying that the enzyme did not recognize the side chain in oriented sterol monolayers [70]. Substrate specificity of a cholesterol oxidase from *Rhodococcus* sp. GK1 was examined and the enzyme was found to be most active with cholesterol (100%), β-sitosterol (70%) and stigmasterol (40%). Sterols with modified A-rings and B-rings or the 3α-OH of cholic acid remained unoxidised [38]. Cholesterol oxidase from *Brevibacterium sterolicum* rapidly oxidized cholesterol, pregnenolone and β-sitosterol but was less reactive towards stigmasterol. However, it was found to be unreactive toward cholic acid, deoxycholic acid, 5α-androstan-3α,17β-diol and androsterone [44]. Except cholesterol, β-cholestanol was also oxidized at a high rate by most enzymes [23]. The double bond in the steroid ring between 5 and 6 positions does not seem to be essential for this enzyme activity and the sterols with the short side chain are oxidized at a low rate comparatively. Although the oxidation rates of pregnenolone by most enzymes were slow, the enzymes from *Chromobacterium* sp. DS-1, *Streptomyces* sp. SA-COO and *S. violascens* oxidized pregnenolone at a high rate [17,71]. *Chromobacterium* sp. DS-1 cholesterol oxidase oxidized most 3β-hydroxysteroids. Interestingly, the cholesterol was the best substrate and β-cholestanol, pregnenolone and β-sitosterol were rapidly oxidized. In contrast, the β-stigmasterol, ergosterol, dehydroepiandrosterone and epiandrosterone were slowly oxidized. Epicholesterol was completely resistant to enzymatic oxidation [17].

Cholesterol is a commonly found steroid with a great importance in biology, medicine and chemistry as it plays an essential role as a structural component of animal cell membranes. Cholesterol is found normally in nature because of its high resistance to microbial degradation.

Owing to its complex spatial conformation and low solubility in water, cholesterol is a very resistant molecule to biodegradation. The high hydrophobicity and low volatility of cholesterol lead to a high absorption to solid phases. Because of high rate of persistence, cholesterol and some derived compounds such as coprostanol have been used as reference biomarkers for environmental pollution analysis [72]. Steroids, some of them derived from cholesterol constitute a new class of pollutants discharged into the environment as a result of human activity [73]. The potent metabolic activities of these compounds affect a large number of cellular processes and thus, their presence and accumulation in water-wastes and in certain ecological niches can affect the endocrine system of animals and humans [74]. Moreover, cholesterol is the main component of lanolin and this and other related sterols are natural contaminants fairly resistant to the anaerobic treatment that is carried out on the effluents from wool-processing industry [75]. In eukaryotes, steroid oxidation and isomerization are important step(s) in the synthesis of a wide variety of steroid hormones that are carried out by NAD^+ dependent 3β- hydroxysteroid dehydrogenase as membrane-bound protein located in the endoplasmic reticulum and mitochondrion [76,77]. Hence flavin-mediated cholesterol oxidation is a process unique to microorganisms. It is generally assumed that the first reaction in the aerobic metabolism of cholesterol is its oxidation to cholestenone through two sequential reactions. That is, the oxidation of cholesterol to cholest-5-en-3-one followed by its isomerization to cholestenone both catalysed by cholesterol oxidase.

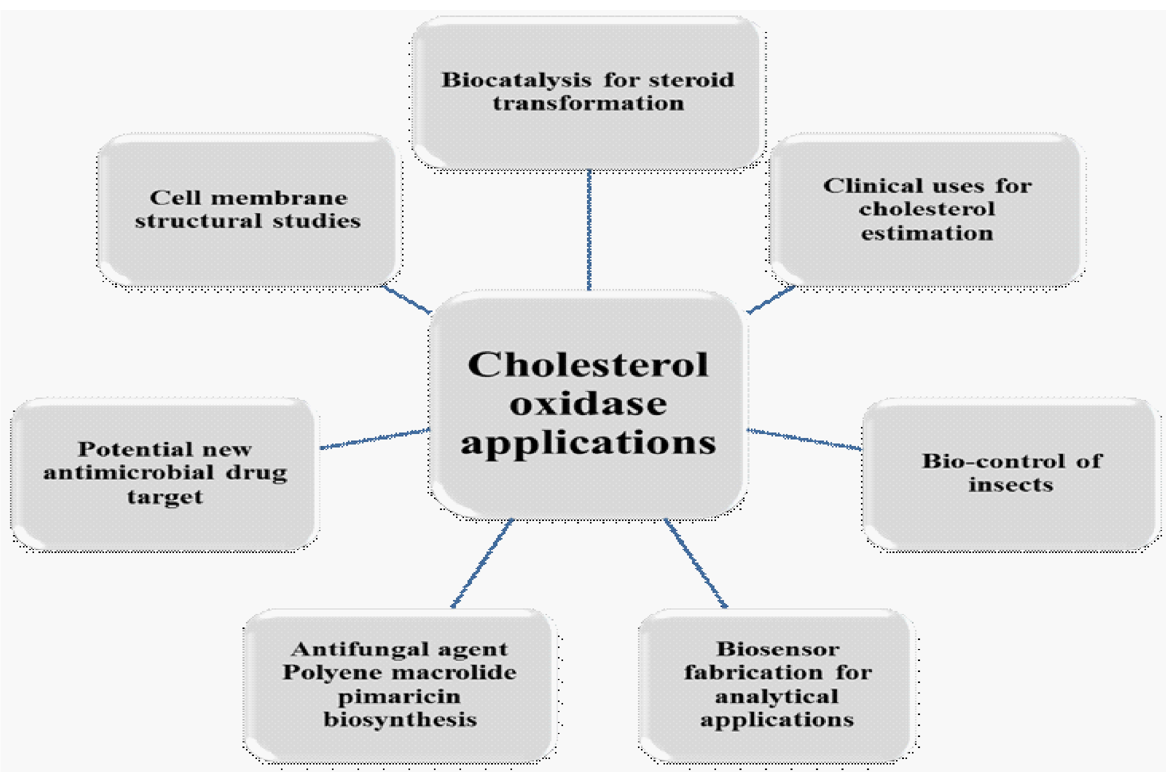

Figure 2: Broader applications of cholesterol oxidase.

5. Major Applications of Cholesterol Oxidase

The microbial oxidases have been reported from many bacterial species and diverse habitats. Invariably, cholesterol oxidases have been exploited in biotransformation's of steroids, biosensors, membrane structural studies, insect control & larvicidal drugs and antimicrobial drugs (**Figure 2**). The broader known applications of cholesterol oxidases are as follows;

5.1. Cholesterol biotransformation

Biotransformation's are structural alterations in a chemical compound which are catalyzed by microorganisms in terms of growing or resting cells or by isolated enzymes. Because of the high stereo- or regio-selectivity combined with high product purity and high enantiomeric excesses, biotransformation's can be technically superior to traditional chemical synthesis. If these features can be combined with economic benefits, biotransformation's become the functional part of new chemical processes for organic synthesis. Further advantages of biocatalytic processes are the mild and ecologically harmless reaction conditions (normal pressure, low temperature and neutral pH), which are important requirements for sustainability. Bioconversions of hydrophobic compounds often meet with two serious obstacles: limited substrate accessibility to the biocatalyst as a result of the low aqueous solubility of most organics; inhibition or toxicity of both substrate and product exerted upon the microorganism [78]. Cholesterol is metabolized by a large number of microorganisms through a complex metabolic pathway involving many enzymatic steps, first step involving the oxidation of the 3β-hydroxyl group followed by the oxidation of the 17- alkyl side chain and the steroid ring system, finally degrading the entire molecule to CO_2 and H_2O [23]. In the sequence of the cholesterol oxidation by microbial cells, 4-cholesten-3-one may be oxidized with accumulation of the steroids AD and ADD, which are important precursors of chemically synthesized hormones or may be transformed to steroid intermediates [79]. *R. equi* DSM 89-133 was employed for the conversion of cholesterol and other sterols to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione and up to 82% of cholesterol was converted when the growth medium was supplemented with acetate [80].

Bioconversion of cholesterol to ADD and AD in cloud point system was studied [81]. Cholesterol was initially converted to cholestenone by *Arthrobacter simplex* U-S-A-18. Cholestenone was prepared directly from the fermentation broth of *A. simplex* and converted to ADD by *Mycobacterium* sp. NRRL B-3683. Conversion of AD to ADD has been reported in mixed culture of *Mycobacterium-Nocardioides* [82]. Biotransformation of sterols is initiated by modification of the 3β-ol-5-ene- to 3-oxo-4-ene moiety. The role of cholesterol oxidase in this process has been elucidated. The cholesterol oxidase is not critical for sterol catabolism in the fast-growing AD-producing *Mycobacterium* sp. VKM Ac-1815D strain and the knock-out of cholesterol oxidase gene did not abrogate sterol ring-A oxidation [83]. Similar conclusions

were made earlier for *Mycobacterium smegmatis* mc2 155 [84]. In *Rhodococcus erythropolis* CECT3014, cholesterol oxidase was shown to be a major inducible extracellular cholesterol oxidase but its disruption did not alter cell growth on cholesterol [85]. However, in *Streptomyces virginiae* IB L-14, inactivation of cholesterol oxidase led to abrogate the oxidation of diosgenin to diosgenone and other 3-oxosteroids [86]. Targeted gene disruption of cholesterol oxidases in *Gordonia cholesterolivorans* CECT 7408T resulted in a mutant strain unable to grow on steroids. Two cholesterol oxidases, ChoM1 and ChoM2 were identified in *Mycobacterium neoaurum* NwIB and these were suggested to be essential for utilization of phytosterol as a carbon source [87]. Along with cholesterol oxidases, 3β-hydroxysteroid dehydrogenases (3β-HSDs) can catalyse 3β-hydroxy group oxidation and $\Delta 5 \rightarrow 4$ isomerization in actinobacteria [84].

Mycobacterium smegmatis PTCC 1307 was used as a microbial agent to produce ADD and AD, two useful precursors in the synthesis of steroid drugs. The side chain of cholesterol, as the substrate, was selectively cleaved in the presence of five enzyme inhibitors. An intermediate structure with intact side chain, cholest-4-ene-3-one, was also detected and purified [88]. Wu and coworkers (2015) explored production of cholest-4-en-3-one by directly using cholesterol oxidase from *Rhodococcus* sp. in an aqueous/organic two-phase system and it was found that the conversion was more efficient in the biphasic system than in the aqueous or co-solvent system. After 48 h of reaction, the conversion rate reached 94.2% in the biphasic system and only 42.3% conversion was achieved in the aqueous system [14]. In another study biotransformation of cholesterol to ADD by cholesterol oxidase from *Chryseobacterium gleum* was studied and the growing cells produced 0.076 g ADD from 1 g cholesterol, which was equivalent to 10% molar conversion of cholesterol [89]. Whole-cells of the recombinant strains *Bacillus subtilis* 168/pMA5-choM1 and *B. subtilis* 168/pMA5-choM2 expressing choM gene encoding cholesterol oxidase from *Mycobacterium neoaurum* JC-12 were used as catalysts for the bioconversion of cholesterol to 4-cholesten-3-one and a percentage conversion of 67% and 83% was observed at 21 h [90]. *R. erythropolis* cholesterol oxidase was employed for the preparative oxidation of cyclic allylic, bicyclic and tricyclic alcohols and for the synthesis of several ergot alkaloids [63].

5.2. Therapeutic uses

Cholesterol oxidase is used as an efficient analytic tool for determining cholesterol in various samples; total and esterified cholesterol in serum or blood, from low-density lipoproteins to high-density lipoproteins, on the cell membrane, in gall stones, in human bile and in various food samples [21]. Normal cholesterol level in human blood is less than 200 mg/dL and lipoproteins contain cholesterol of which ~70% is in esterified form. High levels of cholesterol and cholesteryl esters (hypercholesterolemia) have been associated with cardiovascular disease such as atherosclerosis and other heart diseases, although lower levels (hypocholester-

olemia) may be associated with cancer, depression or respiratory diseases making determining of serum cholesterol concentration very important. Alzheimer disease risk is also reported to be related to hypercholesterolemia *via* involving oxidative stress mechanisms [91]. Cholesterol level in serum is generally determined by using an enzymatic assay [48,69]. As most of cholesterol in serum samples exists in an esterified form so prior incubation of serum with cholesterol esterase (EC 3.1.1.13) is required to release the free cholesterol. Cholesterol oxidase catalyzes oxidation of cholesterol to cholestenone with simultaneous release of H_2O_2. The enzyme subsequently catalyzes the oxidative coupling of $H_2O_2$with a chromogenic dye which is determined spectrophotometrically. In addition to being used in the microanalysis of steroids in food samples it is also used for differentiating the steric configurations of 3-ketosteroids from the corresponding 3β-hydroxysteroids [92]. Over the years, various electrochemical biosensors have been designed by using immobilized cholesterol oxidase for the determination of cholesterol in serum or food samples [93-95].

5.3. Insecticidal activity

Genetically modified plants which are able to control insect pests by producing insecticidal proteins (such as *Bacillus thuringiensis* toxin) are being used very widely to replace the use of synthetic pesticides. A highly efficient bacterial protein capable to kill larvae of boll weevil (*Anthonomus grandis grandis* Boheman) in the culture filtrates of *Streptomyces* sp. was identified as a cholesterol oxidase [15]. When this enzyme was used in purified form against boll weevil larvae, it showed good activity at lethal concentration of 50% (LC_{50} of 20.9 µg.ml), which is well comparable to the insecticidal bioactivity exhibited by *Bacillus thuringiensis* proteins. Cholesterol oxidase insecticidal activity is reported due to induction of lysis at the midgut epithelium of larvae upon ingestion. Cholesterol or some other related sterol at the boll weevil midgut epithelium membrane appeared to be available for oxidation by cholesterol oxidase, causing lysis of the midgut epithelial cells resulting in larval death [23]. The enzyme also showed insecticidal activity against lepidopteran cotton insect pests, including tobacco budworm (*Heliothis virescens*), corn earworm *(Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) [96]. Microbial insecticide proteins are very important in several pest control strategies employed in transgenic crops and cholesterol oxidase gene from *Streptomyces* sp. has been expressed in tobacco protoplasts [97].

5.4. Cell membrane structural studies

Cholesterol is the main sterol constituent of eukaryotic cell membrane essential for maintaining cell membrane structure and stability. The cell surface cholesterol can be determined accurately with cholesterol oxidase hence the surface area of a given cell can be estimated readily [98]. Cholesterol oxidase is used as a probe to investigate cholesterol interaction with phospholipids as cholesterol associates preferentially with sphingolipids in cholesterol-

rich lipid rafts areas of the membranes in eukaryotic cells [100,101]. The lipid rafts are the microdomains in which cholesterol and saturated membrane lipids such as sphingolipids promote the formation of a highly ordered membrane structure in comparison to the more disordered vicinity [102,103]. Lipid rafts play roles in various cellular processes, including signal transduction, protein and lipid sorting, cellular entry by toxins and viruses, and viral budding [23]. Therefore, cholesterol oxidase plays a significant role in the study of the functional aspects of lipid raft with regard to eukaryotic membrane.

5.5. Potential new antimicrobial drug target

A wide variety of microorganisms including some life threatening pathogenic bacteria such as *Rhodococcus equi, Mycobacterium tuberculosis* and *M. leprae* are reported to produce cholesterol oxidase [104]. *Rhodococcus equi* is a Gram-positive bacterium known for causing infection in young horses as well as in humans acting as an opportunistic pathogen in immunocompromised patients [36,105]. Cholesterol oxidase may possibly acts as an interesting pharmaceutical target for treating bacterial infections. *In vitro* studies advocated that during *R. equi* infection of the host cell, membrane lysis is facilitated by the induction of extracellular cholesterol oxidase along with other candidate virulence factors [35]. The membrane-damaging activity of *R. equi* requires the presence of bacterial sphingomyelinase C which suggested that the cholesterol oxidase substrate is not directly accessible to the enzyme in intact membranes [21]. It has also been reported that cholesterol oxidase is also involved in the manifestation of HIV and nonviral prion origin (Alzheimer's) diseases [106]. The pathogenic bacteria utilize cholesterol oxidase for infection by converting the cholesterol of membranes thus causing damage by altering the physical structure of the membrane. The emerging problem of antibiotic resistant bacteria and their abilities for rapid evolution have pushed the need to find alternative antibiotics which are less prone to drug resistance. Since no eukaryotic enzyme homologues exist, this type of bacterial cholesterol oxidase falls into the scope of potential drug target for a new class of antibiotics which still remain to be explored [107].

5.6. Polyene macrolide pimaricin biosynthesis

The *Streptomyces natalensis* cholesterol oxidase which is a product of the *pimE* gene plays an important role in the biosynthesis of the polyene macrolide pimaricin by acting as a signaling molecule [108]. This 26-member tetraene macrolide antifungal antibiotic is used in the food industry to prevent contamination of cheese and other non-sterile food with mold and also for treating the fungal keratitis because of its ability to interacts with membrane sterols especially ergosterol, resulting in the membrane structure alteration that causes leakage of cellular contents [21]. The polyene macrolide pimaricin gene is located in the center of the pimaricin biosynthetic cluster as the pimaricin production is completely blocked by the gene disruption which is recovered after gene complementation. The addition of purified *PimE* or

exogenous cholesterol oxidase to the gene disrupting culture can restore the pimaricin production. These findings suggested the involvement of cholesterol oxidases as signaling proteins for polyene biosynthesis [23].

5.7. Cholesterol oxidase as a novel antitumor therapeutic drug

Cholesterol oxidase catalyzes oxidation of cholesterol and has been used to track membrane cholesterol. Liu and coworkers (2014) reported that cholesterol oxidase from *Bordetella* species made lung cancer cells both *in vitro* and *in vivo* to undergo irreversible apoptosis. Cholesterol oxidase treatment inhibited phosphorylation of Akt (protein kinase B) and ERK1/2 (extracellular signal-regulated kinase 1/2) which was irreversible even after cholesterol addition. Further studies indicated that cholesterol oxidase treatment also promoted the generation of reactive oxygen species (ROS). In addition to this cholesterol oxidase treatment resulted in phosphorylation of JNK (c-Jun NH_2-terminal kinase) and p38, downregulation of Bcl-2 (B-cell lymphoma/leukemia-2), upregulation of Bax with the release of activated caspase-3 and cytochrome C likely due to production of hydrogen peroxide along with cholesterol oxidation. These findings suggested that cholesterol oxidase leads to irreversible cell apoptosis by decreasing cholesterol content and increasing ROS level indicating cholesterol oxidase may be a promising candidate for a novel antitumor therapy [109].

5.8. Probiotics

Probiotics are living microorganisms which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition [110]. Though several *in vitro* and *in vivo* studies have proved that the administration of probiotics decreases serum/plasma total cholesterol, LDL-cholesterol and triglycerides or increases HDL-cholesterol but their hypocholesterolemic effects still remain controversial [111]. The species which have been found to exert cholesterol-lowering effects include genera like *Lactobacillus, Lactococcus, Enterococcus, Streptococcus* and *Bifidobacterium* [112]. Different mechanisms like co-precipitation of cholesterol with deconjugated bile, cholesterol binding to cell walls, integration of cholesterol into the cellular membranes during growth, production of short-chain fatty acids during fermentation and transformation into coprostanol are proposed for cholesterol lowering effects of probiotics [112-115]. Cholesterol oxidase, especially derived from bacterial cells plays a major role in the degradation of cholesterol in many fermented foods [116]. Khiralla, (2015) reported that *Leuconostoc mesenteroides* GMK03 could degrade cholesterol using the extracellular cholesterol oxidase and found to betolerant to low acidity (p^H 2.5) and bile salts (0.3%), competent to adhesion to Caco-2 cells and have low antibiotic resistance, so could be considered as a promising probiotic strain [117]. A gene encoding cholesterol oxidase (choA) from *Streptomyces* [118] has been expressed in several species of probiotic bacteria including *Lactobacillus casei* [119], *Lactobacillus reuteri* [120] and *Streptococcus thermophilus* [121].

Rossi et al., (1998) reported the cloning of cholesterol oxidase from *Streptomyces* sp. into *Bifidobacterium* under its own promoter but without successful expression [122]. Park et al., successfully cloned and expressed cholesterol oxidase gene from a *Streptomyces* sp. in promoter and RBS (ribosome-binding site) of *Bifidobacterium longum* MG1 [123]. Bacterial degradation of cholesterol in cholesterol containing food may be beneficial for human health [124].

5.9. Cholesterol oxidase biosensors for analytical assays

Cholesterol is an important analyte molecule and its assay is important for the diagnosis and prevention of a number of clinical disorders and in food analyses. Moreover, quantification of cholesterol present in various foods is vital for selecting a diet with optimal intake of cholesterol. Thus, it is necessary to develop new techniques for easy and rapid estimation of cholesterol levels in various analytical samples [125]. Biosensors with immobilized enzymes are of huge interest in the various analytical procedures as they facilitate the enzyme reuse along with exclusive selectivity of the biological molecules and the processing power of modern microelectronics [126]. Over a period of time, a variety of cholesterol biosensors have been developed [95]. Most of the reported enzyme-based cholesterol biosensors are fabricated on amperometric technique besides, cholesterol biosensors based on photometric behaviors like luminescence, fluorescence and surface plasmon resonance have also been reported [95,127,128]. Amperometric measurement of O_2 consumption or H_2O_2 production during cholesterol catalysis by cholesterol oxidase is the frequently used strategy in cholesterol biosensors [129]. A cholesterol biosensor based on cholesterol oxidase-poly (diallyldimethyl ammonium chloride)-carbon nanotubes-nickel ferrite nanoparticles (ChOx-PDDACNTs-$NiFe_2O_4$NPs) was fabricated by using a single dropping step on a glassy carbon electrode surface [130]. Various nanoparticles like metal nanoparticles (gold (Au), platinum (Pt), silver (Ag) NPs), metal oxide nanoparticles (zinc oxide (ZnO), iron oxide (Fe_3O_4), cerium oxide (CeO_2), titanium oxide (TiO_2) NPs), carbon nanotubes (single-walled carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs) based materials have also been exploited for designing cholesterol biosensor [131-134].

6. Conclusion

Cholesterol oxidase has been reported from a large number of bacterial species and the actinomycetes being the most common group. Being an enzyme of great commercial value, cholesterol oxidase has drawn significant attention due to its wide spread use in determination of cholesterol level(s) in various clinical and food samples and because of its other novel applications like biocontrol of insects, polyene macrolide pimaricin synthesis, biocatalysis for the synthesis of a number of steroids and fabrication of biosensors. Biochemical and structural studies on cholesterol oxidase can play important roles in understanding of various catalysis aspects of different flavoenzymes as it belongs to flavoprotein oxidases.Thus cholesterol

oxidase is a versatile enzyme, the newer microbial sources in nature need to be explored for synthesis of extracellular cholesterol oxidase(s), which is/ are easy to purify, characterize and put for extended commercial uses.

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Current Research in Microbiology

Chapter 6

Stability, Flexibility, and Function of Dihydrofolate Reductases from *Escherichia coli* and Deep-Sea Bacteria

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Abstract Dihydrofolate reductase (DHFR) is an important target for investigating the linkage between structural flexibility and catalytic function because it is a ubiquitous enzyme existing in all organisms. Site-directed substitution or deletion in flexible loops of *Escherichia coli* DHFR significantly affects the stability, flexibility, and catalytic function, although distal residues have not been recognized as dynamically and functionally significant. Nonadditive effects of double substitution or deletion in different loops demonstrate the existence of long-range coupling between the loop motions that include other distal residues. Compressibility changes due to loop substitutions and ligand binding reveal that the modified flexibility and function can be mainly attributed to changes in internal cavities or atomic packing. DHFRs from deep-sea bacteria exhibit species-specific pressure-dependence on stability and function that provide useful information on the roles of structural flexibility in molecular adaptation to high-pressure environments. These findings give new insight into the structure–flexibility–function relationship of DHFR.

Abbreviations: cAMP: cyclic adenosine monophosphate; CD: circular dichroism; DHF: dihydrofolate; DHFR: dihydrofolate reductase; H/D: hydrogen to deuterium; IPMDH: 3-isopropylmalate dehydrogenase; MD: molecular dynamics; MS: mass spectrometry; MTX: methotrexate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$: oxidized nicotinamide adenine dinucleotide phosphate; NMR: nuclear magnetic resonance; THF: tetrahydrofolate; TOF: time of flight

1. Introduction

Dihydrofolate reductase (DHFR) is an important enzyme that exists ubiquitously in all organisms. DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) by utilizing the reducing cofactor NADPH (reduced nicotinamide adenine dinucleotide phosphate). THF and its derivatives are essential for the syntheses of purine and thymine bases of DNA, and hence DHFR plays a central role in cell growth and proliferation. DHFR is also a clinically important enzyme not only as the target of several antifolate drugs, such as trimethoprim and methotrexate (MTX), but also as an enzyme for producing *l*-leucovorin, an anticancer drug, in a stereospecific manner.

DHFRs from various organisms living in normal and extreme environments have been investigated on the basis of the sequence conservation, backbone flexibility, and enzyme kinetics [1–4]. Among them, DHFR from *Escherichia coli* (ecDHFR) is the most widely investigated because it is a suitable target for investigating the linkage between protein dynamics and catalytic function. The catalytic reaction of ecDHFR is known to occur along a preferred reaction pathway involving several intermediate states that include hydride transfer [5]. The important role of structural dynamics in function of this enzyme was indicated on the basis of various dynamics and catalytic data [6,7]. Sawaya and Kraut [8] proposed a dynamic model for conformational changes during the catalytic cycle based on crystal structures of ecDHFR–ligand complexes analogous to the kinetics intermediates. Studies of mutations within or in the vicinity of the active site [9–14] have uncovered the roles of the residues around the active site, but the roles of those distant from the active site have remained unclear.

The participation of distal residues in catalytic function was demonstrated using various site-directed mutations [15,16], with subsequent studies showing motional couplings between the residues in the active site and the distal residues, which preorganize the Michaelis complex for catalysis [7,17]. A computational simulation showed that the effect of a distal mutation may propagate to active-site residues through modulation of the correlated motion [18]. Although these studies have indicated the significance of distal residues, the relationship between structural flexibility and catalytic function remains controversial because the active-site fluctuation is mediated in a complicated manner by a motional hierarchy of the entire protein molecule, including thermal fluctuation of individual amino acid residues, loop motions, and reorientation of structural domains.

There have also been many studies of DHFRs from microorganisms living in extreme environments of temperature, pressure, salt concentration, pH, etc. in relation to molecular evolution and pharmaceutical applications: hyperthermophilic bacterium *Thermotoga maritima* [19–21], psychrophilic and piezophilic bacterium *Moritella profunda* [22–24], extremely halophilic bacteria *Haloferax volcanii* [25–27] and *Haloarcula japonica* [28,29], and alka-

liphilic bacterium *Bacillus halodurans* [30]. These studies have revealed characteristic species-dependent structures and functions of DHFR, but the molecular mechanisms underlying the adaption to particular environmental conditions remain unclear. Among these extreme environments, the deep sea is of special interest because the various piezophilic, piezosensitive, and piezotolerant species that live there exhibit characteristics that vary with the depth (i.e., hydrostatic pressure).

While conducting such studies of DHFR, we have systematically investigated the effects of loop mutation of ecDHFR and the species dependence of deep-sea DHFRs on stability, flexibility, and function, based on the hypothesis that the atomic packing plays an important role in the overall fluctuation relevant to catalytic function. In 1993 we found that mutations at Gly121 in a loop distant from the active site of ecDHFR affect the stability and function, and proposed that the effects of loop mutation could extend to the entire protein molecule [31]. The subsequent investigations of loop mutations have revealed important roles of loop regions in the dynamics and function of this enzyme [32–36]. Related to the structure–flexibility–function relationship, we have also examined the effects of pressure on the stability and function of DHFRs obtained from various deep-sea bacteria [37–40]. The species-dependent pressure susceptibility of stability and function has provided useful information on the structural flexibility and the molecular adaptation of deep-sea DHFRs to extreme pressure conditions.

The present review focuses on surveying the effects of loop mutations in ecDHFR and the species dependence of deep-sea DHFRs on stability, flexibility, and function, primarily based on our experimental works. These data in combination with molecular dynamics information will deepen our understanding in the structure–flexibility–function relationship and pressure-adaptation mechanism of DHFR, which is complementary to more comprehensive reviews on the related topics [6,7,41–46].

2. Stability, Flexibility, and Function of Loop Mutants of ecDHFR

ecDHFR is a monomeric protein of 159 amino acid residues with no prosthetic group or disulfide bond. **Figure 1** shows the three-dimensional structure of the DHFR–folate–NADP$^+$ (oxidized nicotinamide adenine dinucleotide phosphate) complex in crystal form [8]. It consists of eight β-strands and four α-helices, one of which includes the active site Asp27. The large B-factors suggest the appearance of five flexible loops in DHFR: Met20 (residues 9–23), αC–βC (residues 51–57), βC–βD (residues 64–72), βF–βG (residues 117–131), and βG–βH (residues 142–149) [47]. Nuclear magnetic resonance (NMR) spin relaxation analysis of the ^{15}N-labeled DHFR–folate complex has also revealed four distinctive flexible regions: residues 16–22 in the Met20 loop, residues 67–69 in the βC–βD loop, residues 38 and 88 in the hinge region between the adenosine-binding domain (residues 38–88) and the major domain (residues 1–37 and 89–159), and residues 119–123 in the βF–βG loop [48]. The Met20 loop contacts NADPH

in a closed conformation that is stabilized by hydrogen bonding of Gly15 and Glu17 in the Met20 loop, with Asp122 in the βF–βG loop. A conformational change of the Met20 loop accompanies disruption of these hydrogen bonds to form new ones between Asn23 (Met20 loop) and Ser148 (βG–βH loop). The rearrangement of the hydrogen bond changes the Met20 loop from a closed conformation to the occluded conformation found in the product complex.

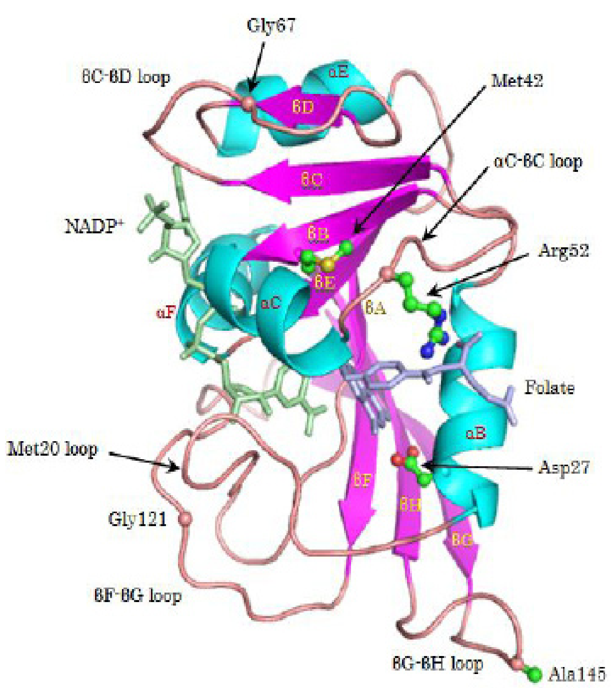

Figure 1: Crystal structure of ecDHFR in a complex with folate and $NADP^+$ (PDB: 1RX2), which represents the Michaelis-complex structure [8]. Residues Met42, Arg52, Gly67, Gly121, and Ala145, which were the mutation targets in this study, and the active-site residue, Asp27, are shown by balls and sticks. The figure was drawn using the program PyMol (http://www.pymol.org/).

The kinetics scheme of the catalytic reaction of ecDHFR has been clarified by Benkovic and coworkers [5,12,49]. As shown in **Figure 2**, ecDHFR catalyzes the reduction of DHF to THF with the aid of coenzyme NADPH *via* five intermediates: DHFR·NADPH, DHFR·NADPH·DHF, DHFR·$NADP^+$·THF, DHFR·THF, and DHFR·NADPH·THF. The rate-determining step at neutral pH is the release of the product (THF) from the reduced ternary complex (DHFR·NADPH·THF). Hydride transfer from NADPH to DHF is the key reaction step that is thought to be regulated by protein motion, the steps of which presumably require kinetically significant conformational changes to occur in the Met20 loop that comes into contact with NADPH prior to catalysis. This enzyme reaction is strongly inhibited by MTX, which is used as an anticancer drug.

A large amount of structural dynamics and catalytic data [8,16–18,48,50–61] including computational simulation [18,51,54,55,57,58,62] have provided a detailed context for the role of dynamics in function of ecDHFR. In this subject, mutation studies in the distal residues [11,15,16,50] in addition to the vicinity of the active site [9,10,12–14] have played important roles in elucidating the motional couplings between the residues in the active site and the distal

residues. Singh et al. experimentally showed the presence of a global dynamic network of coupled motions including residues 42, 121, and 133 correlated to hydride transfer [60]. An NMR study showed the participation of residue 42 in solution dynamics [63]. However, the target residues for mutation are limited and further detailed mutation studies are required for deeper understanding of the precise roles of distal residues or loops in dynamics and function.

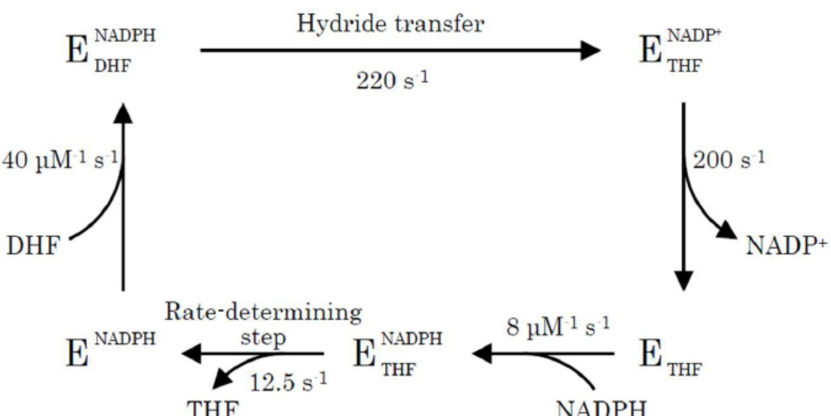

Figure 2: The catalytic cycle of ecDHFR. The five primary intermediates and the rate constants for forward reactions at pH 7 [5,50] are shown. E, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate.

We have systematically investigated the effects of loop mutation of ecDHFR on stability, flexibility, and function to demonstrate the relevance of the flexible loops to catalytic function *via* the participation in overall fluctuation [31–36]. Since the Met20 loop directly contacting NADPH is well characterized, roles of the other four loops (αC–βC, βC–βD, βF–βG, and βG–βH) have been studied using site-directed amino acid substitution or deletion around the most-flexible residues in each loop (Arg52, Gly67, Gly121, and Ala145), which are highly conserved among many DHFR sequences [31–34]. The mutation effects of another pivotal distal residue Met42 were also studied for comparison, because this residue is in the structural core that includes tightly conserved regions (residues 40–43) that do not appear to play any obvious functional roles in the crystal structure [64].

2.1. Effects of loop mutations on stability

The structural stability is evaluated by the difference in the Gibbs free energy between native and denatured states (ΔG°_u) when the denaturant (urea or guanidine hydrochloride) is at an infinite dilution:

$$\Delta G_u = \Delta G^\circ_u - m\,[\text{denaturant}] \quad (1)$$

where ΔG_u is the change in free energy due to denaturation at a given denaturant concentration, and coefficient m is the cooperativity parameter of the transition, which is dominantly attributable to the difference in the solvent-exposed surface area of a protein molecule between the

native and denatured states [65–67]. C_m is the denaturant concentration at the midpoint of the transition (i.e., $\Delta G_u = 0$).

2.1.1. Loop-residue substitution

The $\Delta G°_u$, m, and C_m values for urea denaturation of substitution mutants at residues 67, 121, and 145 are listed in **Table 1** [32–34]. It is evident that the $\Delta G°_u$ values of any mutants differ from that of the wild type: $\Delta G°_u$ decreased for most mutants at residues 67 and 121 whereas it increased for most mutants at residue 145, indicating that the loops contribute to the stability in different ways. The m value was also dependent on the mutations at any residues. The $\Delta G°_u$ value increased with the m value, with correlation coefficients (r values) of 0.80, 0.72, and 0.97 for residues 67, 121, and 145, respectively. This indicates that the stability increases with the cooperativity of the transitions or the compactness of the native state.

A particularly interesting observation was that the $\Delta G°_u$ value decreased as the hydrophobicity of the introduced amino acid side chains increased. Such a reverse hydrophobic effect could be due to the denatured state being stabilized and/or the native state being destabilized by the hydrophobic interaction. The former mechanism would dominate for a mutation at residue 145, which is highly exposed to the solvent, whereas the latter would dominate for mutations at residues 67 and 121, because the $\Delta G°_u$ value decreased as the volume of the amino acid side chain introduced into both residues increased: the bulky side chain would affect the atomic packing to overcome the hydrophobic stabilization effect. In contrast to the loop mutations, the $\Delta G°_u$ values of Met42 mutants (which are also listed in **Table 1**) increased with the side-chain hydrophobicity [64]. This inverted correlation seems reasonable because Met42 is located in the hydrophobic core of the structure. The results that the stability is affected by only a single amino acid substitution in loops or distal residues suggest that the effects of the mutation extend over a long distance and so can modify the structural flexibility.

Double-mutation analyses are useful for exploring the possible couplings between distant loops [68]. The $\Delta G°_u$ values of eight double-substitution mutants at residues 67 and 121 (G67V/G121S, G67V/G121A, G67V/G121C, G67V/G121D, G67V/G121V, G67V/G121H, G67V/G121L, and G67V/G121Y) are listed in **Table 1** in comparison with those of the corresponding single substitution mutants [35]. The $\Delta G°_u$ values of these double mutants (with the exception of G67V/G121H) did not equal the sum of those of the respective single mutants (**Figure 3**). This nonadditivity represents evidence of the presence of long-range interactions between residues 67 and 121, whose α-carbons are separated by 27.7 Å. Thus, the effects of mutation at residue 67 reach residue 121, and *vice versa*. A small alteration in atomic packing due to a mutation at each site would be cooperatively magnified through motional coupling of the loops and thereby affect the structural stability.

Table 1: Thermodynamic parameters for urea denaturation ($\Delta G°_u$, C_m, and m), partial specific volume (v^o), compressibility (β_s^o), and steady-state kinetics parameters (K_m and k_{cat}) of the wild-type and site-directed substitution/deletion mutants of ecDHFR[a].

DHFR	$\Delta G°_u$ (kcal mol^{-1})	C_m (M)	m (kcal mol^{-1} M^{-1})	v^o (cm^3 g^{-1})	β_s^o (Mbar^{-1})	K_m (μM)	k_{cat} (s^{-1})
Wild-type	6.08±0.18	3.11	1.96±0.06	0.723±0.001	1.7±0.3	1.3±0.1	24.6±3.1
M42E	3.07±0.28	1.57	1.96±0.15	ND	ND	6.0±0.7	15.9±0.4
M42S	4.50±0.29	2.42	1.86±0.11	ND	ND	2.3±0.2	22.6±0.6
M42Q	4.37±0.22	2.12	2.06±0.10	ND	ND	2.3±0.2	24.3±0.8
M42G	4.40±0.50	1.93	2.28±0.24	ND	ND	1.2±0.1	13.0±0.4
M42T	2.90±0.39	1.90	1.53±0.17	ND	ND	0.8±0.1	18.7±0.4
M42A	5.75±0.54	2.35	2.45±0.22	ND	ND	1.3±0.2	14.0±0.8
M42H	3.06±0.43	1.57	1.95±0.22	ND	ND	1.9±0.3	26.6±1.5
M42P	4.78±1.10	3.27	1.46±0.36	ND	ND	0.8±0.1	24.0±1.1
M42C	4.81±0.26	2.29	2.10±0.11	ND	ND	1.9±0.2	30.4±1.2
M42V	5.04±0.38	2.41	2.09±0.15	ND	ND	0.9±0.1	15.7±0.7
M42L	5.34±0.51	2.52	2.12±0.19	ND	ND	1.1±0.1	18.9±0.6
M42I	5.85±0.58	2.70	2.17±0.21	ND	ND	0.6±0.1	12.4±0.5
M42Y	6.40±0.48	2.51	2.55±0.18	ND	ND	1.8±0.2	45.5±1.3
M42W	4.17±0.40	2.88	1.45±0.14	ND	ND	45.0±2.6	104.6±2.3
G67S	6.15±0.18	2.82	2.18±0.06	0.723±0.003	1.9±0.8	1.4±0.1	26.4±1.3
G67A	6.60±0.08	2.82	2.34±0.03	0.721±0.002	–0.1±0.9	1.1±0.1	20.0±0.7
G67C	5.72±0.12	2.79	2.05±0.04	0.724±0.001	1.7±0.3	1.2±0.1	24.6±3.6
G67D	5.94±0.12	2.62	2.27±0.04	0.724±0.003	3.0±0.7	1.2±0.2	20.6±2.5
G67V	5.14±0.17	2.89	1.78±0.05	0.721±0.003	1.1±0.5	1.3±0.5	19.1±1.7
G67L	5.64±0.17	2.83	1.99±0.06	0.721±0.003	–0.6±1.4	1.1±0.1	22.6±3.7
G67T	5.87±0.05	2.62	2.24±0.05	0.718±0.002	1.4±1.5	1.8±0.4	26.6±3.1
G121S	6.09±0.16	3.03	2.01±0.05	0.721±0.002	0.7±1.0	1.8	5.2
G121A	6.58±0.17	2.86	2.30±0.06	0.710±0.003	–0.4±0.4	2.7	8.5
G121C	6.17±0.22	2.86	2.16±0.07	0.725±0.001	5.5±0.5	1.5	8.4
G121D	6.27±0.15	2.70	2.32±0.03	ND	ND	1.6	0.67
G121V	5.09±0.20	2.65	1.92±0.08	0.733±0.003	3.7±2.6	1.4	0.94
G121L	5.45±0.17	2.78	1.96±0.06	ND	ND	2.3	0.94
G121H	5.97±0.23	2.64	2.26±0.09	0.713±0.002	–1.8±0.5	2.2	4.8
G121Y	5.88±0.18	2.72	2.16±0.06	0.724±0.001	–0.7±0.6	2.5	4.4
A145G	7.38±0.22	3.05	2.42±0.07	0.726±0.001	3.1±0.2	1.2±0.1	21.1±2.4
A145S	7.28±0.26	3.05	2.38±0.09	0.729±0.002	3.1±0.8	0.8±0.0	19.3±0.5
A145T	9.13±0.26	3.10	2.95±0.08	0.729±0.001	3.0±0.3	1.6±0.1	20.0±0.1
A145V	6.45±0.20	2.85	2.27±0.07	ND	ND	1.1±0.2	15.2±1.5

A145H	7.25±0.21	2.97	2.44±0.07	0.728±0.001	3.8±0.6	0.7±0.2	18.8±0.8
A145F	5.94±0.17	2.96	2.01±0.06	ND	ND	1.2±0.3	27.6±7.9
A145R	8.05±0.23	3.26	2.48±0.07	0.725±0.002	0.8±0.1	1.4±0.2	19.9±1.5
G67V/ G121S	5.86±0.14	2.72	2.15±0.05	ND	ND	2.1±0.6	1.1±0.1
G67V/ G121A	6.73±0.14	3.36	2.00±0.04	ND	ND	1.1±0.3	3.5±0.2
G67V/ G121C	4.78±0.13	2.69	1.77±0.04	ND	ND	2.0±0.5	1.5±0.1
G67V/ G121D	4.77±0.13	2.50	1.91±0.05	ND	ND	2.4±1.0	0.3±0.1
G67V/ G121V	5.15±0.10	2.51	2.05±0.04	ND	ND	3.3±0.7	0.7±0.1
G67V/ G121H	5.05±0.13	2.51	2.01±0.05	ND	ND	1.4±0.7	1.6±0.3
G67V/ G121L	5.07±0.14	2.73	1.85±0.05	ND	ND	2.0±0.9	2.0±0.3
G67V/ G121Y	7.08±0.17	3.24	2.19±0.06	ND	ND	2.6±0.8	1.0±0.1
Δ52	6.52±0.40	3.24±0.29	2.01±0.13	ND	ND	116.1±17.2	96.3±10.9
Δ67	4.27±0.26	1.93±0.16	2.21±0.12	ND	ND	5.1±0.4	16.5±0.4
Δ121	5.16±0.28	2.49±0.19	2.07±0.11	ND	ND	10.8±0.8	1.7±0.1
Δ145	4.86±0.42	2.35±0.27	2.07±0.16	ND	ND	0.8±0.1	24.0±0.4
Δ52/ Δ67	5.43±0.71	2.23±0.40	2.43±0.30	ND	ND	50.2±5.7	23.1±1.2
Δ52/ Δ121	4.09±0.57	2.32±0.44	1.76±0.23	ND	ND	51.2±2.8	0.37±0.01
Δ52/ Δ145	3.25±0.45	1.95±0.35	1.67±0.19	ND	ND	77.7±11.7	31.9±2.5
Δ67/ Δ121	ND	ND	ND	ND	ND	5.6±0.7	0.38±0.02
Δ67/ Δ145	ND	ND	ND	ND	ND	4.8±0.1	41.1±0.4
Δ121/ Δ145	3.21±0.78	1.89±0.59	1.70±0.34	ND	ND	7.3±0.4	0.22±0.00

[a] ΔG°_{u}, C_m, m, v^o, and $\beta_s^{\,o}$ values were measured at pH 7.0 and 15°C. K_m and k_{cat} values were measured at pH 7.0 and 25°C. Taken from Gekko et al. [31,32,77], Ohmae et al. [33–35,64], and Horiuchi et al. [72].
ND, not determined. Data are mean±SD values.

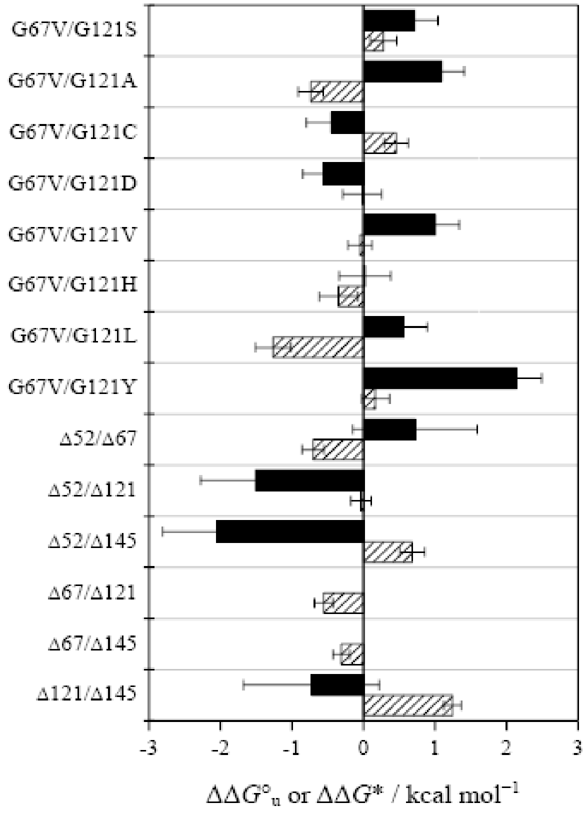

Figure 3: Nonadditive effects of double mutations in flexible loops on stability, $\Delta\Delta G°_u$ (filled bars), and enzyme function, $\Delta\Delta G^*$ (hatched bars). These values were calculated using the following equations with data in **Table 1**:

$\Delta\Delta G°_u = (\Delta G°_{u,\ X/Y} - \Delta G°_{u,\ X}) - (\Delta G°_{u,\ Y} - \Delta G°_{u,\ \text{wild-type}})$

$\Delta\Delta G^* = RT \ln [\{(k_{cat}/K_m)_X \cdot (k_{cat}/K_m)_Y\}/\{(k_{cat}/K_m)_{X/Y} \cdot (k_{cat}/K_m)_{\text{wild-type}}\}]$

where the subscripts X and Y attached to $\Delta G°_u$ and k_{cat}/K_m denote the single mutants and X/Y denotes the corresponding double mutant [35].

The thermal stability of ecDHFR is also influenced by loop mutations [31,32,69]. The thermal transition temperatures of eight mutants at residue 121 were within the range of 43.8–46.9°C, and so were lower than that of the wild type (49.3°C). The calorimetric enthalpy of denaturation also decreased, and so the destabilization of these mutants was attributed to an enthalpy effect rather than an entropy effect. In most mutants, the ratios of the van't Hoff enthalpy to the calorimetric enthalpy were smaller than unity (actually around 0.5), suggesting that the thermal denaturation cannot be explained by a two-state unfolding mechanism and that at least one intermediate exists during the process of thermal denaturation [32,69,70]. Such an intermediate was also observed in the acid denaturation of wild-type ecDHFR around a pH of 4 at 15°C [70]. Judging from the similarity of the circular dichroism (CD) spectra, the intermediates found in both types of denaturation may be a molten globule such as that involved in the folding kinetics [71].

2.1.2. Loop-residue deletion

Since the deletion of a loop residue shortens the loop and reduces flexibility, a deletion mutation is expected to have greater structural and dynamical impacts on the corresponding part than does a substitutive mutation, and hence will amplify the effects caused by distal mu-

tations and so make the mutual couplings among the distant residues more apparent.

The values of ΔG°_{u}, m, and C_{m} for deletion mutants at residues 52, 67, 121, and 145, which are denoted as Δ52, Δ67, Δ121, and Δ145, respectively, are listed in **Table 1** [72]. These deletion mutants showed marked reductions in stability but only marginal changes in *m* values. The small changes in *m* values suggest that the solvent-accessible surface areas in the mutant proteins do not differ markedly from that of the wild type. Δ67, Δ121, and Δ145 showed more significant reductions in structural stability than did those for the corresponding substitution mutants, although the changes in *m* values for these mutants were not significant as the changes observed for the corresponding substitution mutants (**Table 1**). Therefore, the destabilization induced by deleting these loop residues may be attributed to changes in flexibility of the loops as suggested by the findings of substitutive mutation studies [32–34]. In contrast to the other three deletion mutants, Δ52 retained stability comparable to that of wild type, and its change in *m* value was also small, although deleting residue 52 may alter the flexibility of the αC–βC loop by pinching this loop. This finding suggests that deleting this residue caused little structural change, as expected from the small changes in the CD spectrum. The marginal stability change in Δ52 may be due to Arg52 being an ionic residue that is highly exposed to the solvent, although the other three residues tested are all hydrophobic and exhibit somewhat varied structures. In spite of the small change in the stability of Δ52, this deletion caused significant functional changes [72].

The possibility of couplings between the distal loops was also explored using double-deletion mutants (**Figure 3**). Considering experimental errors, Δ52/Δ67 and Δ121/Δ145 showed additive decreases in stability, indicating the absence of any apparent coupling between the αC–βC and βC–βD loops or between the βF–βG and βG–βH loops regarding structural stability. On the other hand, Δ52/Δ121 and Δ52/Δ145 showed nonadditive or synergistic destabilization: the motional and structural change in the αC–βC loop resulting from deleting residue 52 enhances the destabilization by Δ121 or Δ145, probably through modulating the motional correlation between the corresponding loops. This implicates the existence of interplay between the αC–βC loop and the βF–βG or βG–βH loop that modulates the structural stability.

2.2. Effects of loop mutations on flexibility

The protein dynamics of ecDHFR and its mutants have been investigated using various techniques aimed at determining their magnitude and timescales: the B-factor of X-ray crystallography [8,47,73], order parameter of NMR [48,63,74,75], fluorescence relaxation time [16], compressibility [76–79], and MD simulations [18,51,54,62]. Among these techniques, compressibility gives unique information on the volume fluctuation of the protein structure although it does not provide atomic resolution or timescale information, in contrast to X-ray crystallography, NMR, and fluorescence spectroscopy. Only small changes have often been

detected in the X-ray crystal structures of many proteins, even in cases where ligand binding and mutation clearly induce large differences in function and stability. However, the compressibility sensitively reflects modification of the internal atomic packing due to such local structure changes. Thus, the compressibility changes due to ligand binding [78] and mutation [76, 77] give useful information for understanding the structure–flexibility–function relationships of a protein, although such data are scarce due to the associated experimental difficulties.

2.2.1. Compressibility

There are many packing defects (cavities) in the interior of a protein molecule that allow internal motions or flexibility to thermal or mechanical forces. Such cavities are easily compressed by pressure, and hence compressibility is an important measure of protein dynamics and volume fluctuation. According to statistical thermodynamics, the volume fluctuation $<\Delta V_M^2>$ of a molecule with volume V_M is related to its isothermal compressibility coefficient $\beta_{T,M}$ [80] according to

$$<\Delta V_M^2> = k_B T V_M \beta_{T,M} \tag{2}$$

where k_B is the Boltzmann constant and T is the absolute temperature. The volume fluctuation of a protein molecule in solution may be estimated by assuming an analogous equation in which V_M and $\beta_{T,M}$ are replaced by the corresponding partial quantities [79,81]. The coefficient of partial specific compressibility of a protein molecule in solution, β^o, is defined as the pressure derivative of the partial specific volume (v^o), which consists of three contributions: the constitutive atomic volume (v_c), the cavity volume (v_{cav}), and the volume change due to solvation or hydration (Δv_{sol}). Since the constitutive atom is assumed to be incompressible, the experimentally observed β^o value of a protein can be mainly attributed to the pressure effects on two volumetric terms, v_{cav} and Δv_{sol} [79,81]:

$$v^o = v_c + v_{cav} + \Delta v_{sol} \tag{3}$$

$$\beta^o = -(1/v^o)(\partial v^o/\partial P) = -(1/v^o)[(\partial v_{cav}/\partial P) + (\partial \Delta v_{sol}/\partial P)] \tag{4}$$

The first and second terms on the right-hand side of Eq. 4 contribute positively and negatively, respectively, to β^o, and so β^o is sensitively affected by the protein structure. Although direct measurement of isothermal compressibility is technically difficult for proteins, adiabatic one β_s^o can be determined using precise sound velocity and density measurements. The β_s^o values have been measured for more than 50 proteins in water or dilute buffer solutions by many groups, with the largest set of β_s^o data of proteins including mutants having been reported by Gekko and coworkers [77,79,81].

The v^o and β_s^o values of various substitution mutants at residues 67, 121, and 145 are listed in **Table 1** [77]. A particularly interesting finding was that these loop mutations induce

large changes in v^o (0.710 to 0.733 $cm^3\ g^{-1}$) and $\beta_s{}^o$ (–1.8 to 5.5 $Mbar^{-1}$) from the corresponding values of wild-type enzyme (v^o = 0.723 $cm^3\ g^{-1}$, $\beta_s{}^o$ = 1.7 $Mbar^{-1}$). Since the amount of hydration and constitutive atomic volume should be hardly affected by substituting 1 of 159 amino acid residues, these changes in $\beta_s{}^o$ and v^o could be dominantly attributed to modifications of internal cavities.

The positive correlation (r = 0.70) between $\beta_s{}^o$ and v^o supports that cavities contribute significantly to the changes in flexibility induced by mutations. The $\beta_s{}^o$ value tends to decrease with the increased volume of an introduced amino acid (r = –0.66), and hence the structural flexibility seems to be reduced by introducing a bulky side chain. This implies that the loop mutations affect not only the local atomic packing around the mutation sites, but also the internal cavities throughout the protein molecule *via* long-range interaction effects. We observed significant changes in the B-factors of the main-chain atoms and the cavities at positions far from mutation residues 67 and 121 (unpublished data). Moreover, computer simulations predicted that Gly67 and Gly121 cannot be replaced by any other amino acid residues without accompanying movements of the backbone polypeptide chain. Thus, a single amino acid substitution in these loops dramatically influences the flexibility of ecDHFR by modifying the atomic packing, which leads to modification of the enzyme function. A high-pressure NMR study of folate-bound ecDHFR revealed that these loop regions are greatly affected by pressure as well as hinge motion of the Met20 loop: the $^{15}N/^{1}H$-HSQC (heteronuclear single quantum coherence) spectra at 200 MPa showed a significant decrease in cross-peak intensity for residues 5, 12, 15, 23, 37, 52, 54, 67, 79, and 124, and a splitting of signals for residues 12, 13, 22, 51, and 95 [75]. These data support that loop regions play important roles in protein dynamics and function *via* atomic packing. Since no definite correlation was found between $\beta_s{}^o$ and $\Delta G°_u$, the rigid mutant is not necessarily stable against urea.

2.2.2. Hydrogen/deuterium exchange

The exchange of amide hydrogen to deuterium (H/D exchange) of the polypeptide backbone has been widely used as a measure of protein dynamics because the rate and number of exchangeable amide hydrogen atoms can be determined concomitantly. The H/D exchange kinetics of a protein has been explained by the local-unfolding model or EX_2 mechanism in which the rate of exchange (k_{ex}) is determined by the transient opening of the folded structure [82,83]. Although H/D exchange has often been monitored by infrared spectroscopy and NMR [84,85], recent developments in mass spectrometry (MS) have provided a new method for analyzing the H/D exchange of proteins [82,86,87].

The H/D exchange kinetics of ecDHFR have been studied using matrix-assisted laser desorption/ionization time-of-flight (TOF) MS analysis combined with pepsin digestion to elucidate the backbone-fluctuation map [88]. The 18 digestion fragments covering almost

the entire amino acid sequence exhibited significant variations in k_{ex} (0.47–0.71 min^{-1}), in the fraction of deuterium incorporation at the initial stage (D_o = 0.20–0.60), in the fraction of deuterium incorporation at infinite time (D_∞ = 0.75–0.97), and in the number of hydrogen atoms protected from exchange (N_p = 0.4–4.7) relative to the corresponding values for the entire ecDHFR molecule (k_{ex} = 0.51 min^{-1}, D_o = 0.41, D_∞= 0.85, and N_p= 20.7). The H/D exchange process was very rapid in the fragment comprising residues 5–28 (Met20 loop), rapid in disordered and hydrophobic fragments, but slow in β-strand-rich fragments. These results indicate that each fragment makes a different contribution to the fluctuations of the ecDHFR molecule.

The H/D exchange kinetics parameters of the substitution mutants at residues 67 and 121 were also determined by electrospray ionization TOF MS [89]. These mutations induced significant changes in k_{ex} (0.10–0.27 min^{-1}), in the number of fast-exchangeable hydrogen atoms (ΔM_o = 164–222 Da), and in the number of hydrogen atoms protected from exchange (ΔM_∞ = 15–56 Da) relative to the corresponding values for the wild-type enzyme (k_{ex} = 0.18 min^{-1}, ΔM_o = 164 Da, and ΔM_∞= 50.5 Da). These kinetics parameters were strongly correlated with the volume of introduced amino acids and weakly correlated with β_s^o and ΔG°_u. Thus, H/D exchange data as obtained using MS also support that the loop mutations significantly affect the structural fluctuations of the entire molecule.

2.3. Effects of loop mutations on function

As shown in **Figure 2**, ecDHFR catalyzes the reduction of DHF to THF with the aid of coenzyme NADPH *via* five intermediates: DHFR·NADPH, DHFR·NADPH·DHF, DHFR·$NADP^+$·THF, DHFR·THF, and DHFR·NADPH·THF. The effects of mutation on these steps have been examined *via* steady-state and pre-steady state kinetics measurements [16, 49]. The rate constants for ligand binding or releasing, most of which have been measured by Benkovic's group, are important to better understand the catalytic mechanism of ecDHFR and its mutants. Intrinsic kinetic isotope effects on hydride transfer have revealed the correlation of network motions to catalytic reaction [45,60,61]. Although the steady-state kinetics based on the overall rate-determining step cannot present such detailed information on the modified steps in catalytic reaction, they are valuable for comparatively diagnosing the effects of mutation on the catalytic function.

2.3.1. Loop-residue substitution

The steady-state kinetics parameters K_m and k_{cat} for the substitution mutants at residues 42, 67, 121, and 145 are listed in **Table 1** [31–34,64]. It is evident that these parameters are dependent on the mutants, resulting in a definite modification of the enzyme activity (k_{cat}/K_m). It is unlikely that these distal residues participate directly in the enzyme reaction because the α-carbons of Gly67, Gly121, and Alal45 are 29.3, 19.0, and 14.4 Å, respectively, from the

catalytic residue Asp27, and their shortest distances from the NADPH molecule are 8.5, 10.6, and 29.2 Å. The Gly121 mutant was the one that showed significantly lower k_{cat}/K_m values than the wild-type enzyme (at most a 42-fold decrease for G121L), primarily due to a reduced turnover rate (k_{cat}) and little change in the substrate-binding ability (K_m) [31,32]. Although to our knowledge, this was the first evidence for Gly121 participating in the function of ecDHFR, it was difficult to identify the affected steps within the catalytic cycle from knowledge of the steady-state kinetics only. Cameron and Benkovic [50] used a stopped-flow analysis to reveal that G121V results in a 40-fold decrease in the NADPH-binding affinity, a 200-fold decrease in the hydride transfer rate, and a 7-fold decrease in the rate of product release. Furthermore, this mutation introduced a new step into the catalytic cycle that reflects a slow conformational change prior to hydride transfer and probably involves exchange of the nicotinamide ring of NADPH into the active site to form the Michaelis complex [50]. Many subsequent experimental and theoretical studies have demonstrated the important roles of this residue in dynamics and function of ecDHFR [17,59,60,69,90–93].

In contrast to Gly121 mutants, mutations at residues 67 and 145 caused marginal disturbance to enzymatic catalysis, although they significantly changed the structural stability (**Table 1**) [33,34]. The flexibility of Gly67 has been found to be functionally irrelevant [17], but this residue showed motional correlation with the Met20 loop in the MD simulations of the Michaelis complex (DHFR·NADPH·DHF), as found for Gly121 [54,56,69]. According to the explanation for the effects of Gly121 on the enzyme reaction, Gly67 is anticipated to disturb catalysis somewhat. The rate of hydride transfer from NADPH to DHF was influenced by mutations at residue 67, although K_m and k_{cat} were influenced only slightly; hydride transfer was fully rate-determining for G67C and G67D while only partially rate-determining for G67S, G67L, and G67T [33]. On the other hand, Ala145 did not show any apparent motional correlation with the residues in the Met20 loop, but it was correlated with the residues around Met42 that are strongly correlated with the Met20 loop [18]. Therefore, Ala145 mutants would be expected to indirectly disturb catalysis *via* the motional coupling with Met42.

Both the K_m and k_{cat} values of Met42 mutants increased with the side-chain hydrophobicity, with the M42W mutant showing exceptionally large increases in K_m (35-fold) and k_{cat} (4.3-fold) relative to the wild-type enzyme [64]. Such significant effects of Met42 mutants might be consistent with MD simulations of the Michaelis complex showing correlated motion between the regions containing Met42 and Gly121 [18, 60]. Thus, Met42 is an important distal residue for the function of this enzyme, and is correlated with the dynamics of the Met20 and other loops.

Double-substitution mutants at residues 67 and 121 exhibited only small changes in K_m but large changes in k_{cat} (**Table 1**). These changes could be mainly accounted for by Gly121-inducing effects. The transition-state stabilization energy defined as $-RT\ln(k_{cat}/K_m)$ of G67V/

G121A, G67V/G121C, and G67V/G121L was obviously not equal to the sum of the values for the corresponding single mutants (**Figure 3**) [35]. Thus, the additivity rule does not hold for these double mutants, and there exist long-range interactions (motional coupling) between both residues, as found for the stability of double mutants. Double mutants at residues 42 and 121 also showed nonadditive or synergistic decreases in hydride transfer rates with small or negligible changes in other enzymatic parameters [17], suggesting that the two distal residues are coupled in dynamic processes so as to organize the Michaelis complex into the active form.

2.3.2. Loop-residue deletion

The steady-state kinetics parameters K_m and k_{cat} for Δ52, Δ67, Δ121, and Δ145 mutants are listed in **Table 1** [72]. It is evident that deleting these residues induced changes in K_m and k_{cat}, resulting in a definite modification of the enzyme activity (k_{cat}/K_m) as was also found for substitutive mutations. Marked reductions in both K_m and k_{cat} of Δ121 are consistent with Miller and Benkovic [90] finding that in contrast to the substitutive mutation of Gly121, the deletion of Gly121 dramatically decreased the rate of hydride transfer (by 550-fold) and the cofactor-binding strengths for NADPH and $NADP^+$ (by 20-fold and 7-fold, respectively). The marked reduction in the hydride transfer rate can be attributed to the deformed Michaelis complex of Δ121, which may have poorly coordinated ligands for the enzymatic reaction. These results for Δ121 have demonstrated the important dynamic role of the βF–βG loop in ecDHFR catalysis through its greater structural perturbation compared to the substitutive mutation of Gly121 [17,50].

Δ67 showed greatly reduced activity compared to the corresponding substitution mutants, suggesting that deletion of Gly67 has a greater impact on the βC–βD loop than does amino acid replacement (**Table 1**). Since Δ67 showed an apparent stability reduction without any gross structural change, as judged from its *m* value and CD spectrum, the reduced activity could hardly be attributed to structural deformation. Similar to the effects of substitutive mutation at residue 121, the effect of deleting residue 67 may be due to motional coupling between the βC–βD and Met20 loops, which was predicted by MD simulations [18].

Δ145 had unexpectedly greater activity than the wild type mainly due to the decreased K_m, since k_{cat} remained at almost the same level as in the wild type. Δ145 also showed reduced structural stability, but with no apparent overall structural change revealed by the CD spectrum. The deletion of Ala145 may induce motional or conformational changes of the βG–βH loop, or both types of changes, that propagate to the DHF-binding site and enhance its binding. The motional correlation map simulated by MD for the Michaelis complex revealed only a slight correlation between the βG–βH loop and the αB helix (residues 24–35), which is a binding scaffold for DHF [18]. This result suggests that the increased affinity of DHF could be

attributable to structural rearrangement around the DHF-binding site, although a change in the dynamics at the residue cannot be ruled out since Ala145 exhibits a motional correlation with the residues around Met42 that are strongly correlated with the Met20 loop. This highlights the spatial proximity of Ala145 to the DHF-binding site.

Δ52 has another remarkable feature of a 100-fold reduction in ligand-binding strength and a 4-fold increase in k_{cat}. Since the αC–βC loop is spatially close to DHF in the Michaelis complex (**Figure 1**), the large changes in K_m may be explained by the depletion of ionic interactions between DHF and Arg52, while the changes in k_{cat} may be explained by dynamic and conformational changes induced by deleting this residue. However, the marked changes in k_{cat} of Δ52 cannot be readily explained using the available dynamic data because the αC–βC loop does not appear to be correlated with the other regions of the protein in the MD-derived motional correlation map. Although the αC–βC loop has not been focused, it could be another hotspot that modulates enzymatic activity. Deletion of two residues 45 and 46 in the loop region (residues 40–46) in human DHFR has also been shown to affect stability and function *via* the modification of structural flexibility [94].

In all of the double-deletion mutants tested (Δ52/Δ67, Δ52/Δ121, Δ52/Δ145, Δ67/Δ121, Δ67/Δ145, and Δ121/Δ145), the effect of a second mutation was found to be nonadditive except for Δ52/Δ121, with prominent nonadditivity for Δ121/Δ145 and marginal effects for Δ67/Δ145 (**Figure 3**). The loops containing Gly121 and Ala145 are both engaged in hydride transfer and product release in the wild type. The significant nonadditive effect found for Δ121/Δ145 could therefore be explained by the Met20 loop changing the hydrogen-bonding partner from βF–βG to βG–βH loops harboring the respective residues 121 and 145, according to the enzyme reaction [8]. It should be noted that Δ121/Δ145 showed an additive effect on the structural stability but a nonadditive effect on the function. This is possible because the stability is related to the free energies of both the native and denatured states, whereas the enzyme function is only relevant to the native state. Δ67/Δ121 also showed more significant effects than the double-substitution mutants between residues 67 and 121. Although the magnitudes of the effects differed, the statistically observed significant nonadditivity for all of the combinations of deletion residues clearly indicates that the loops tested have functional interdependence.

2.3.3. Relationship between flexibility and function

The relationships between structural dynamics and catalytic function of ecDHFR have been discussed in many experimental and theoretical investigations as summarized in recent reviews [42–46]. These studies have revealed or predicted the network of coupled motions including various distal residues correlated to catalytic function, demonstrating the significance of the distal residues. An interesting problem is whether the overall structural flexibility of ecDHFR takes part in the catalytic function.

A comparison between β_s^o and the steady-state kinetics parameters gives useful information on the flexibility–function relationship, although β_s^o gives no motional features of the fluctuating enzyme. **Figure 4** shows plots of the β_s^o values of kinetics intermediates in the reaction pathway, in which the β_s^o value of the transient DHFR·NADPH·DHF complex—which is unobtainable as a stable complex—was assumed to be the same as that of the ternary complex DHFR·NADPH·MTX based on the structural similarity of the two ternary complexes [78]. β_s^o changes when the coenzyme and substrate are bound or released, while the transient state DHFR·NADPH·DHF is the most flexible and the DHFR·$NADP^+$·THF is the most rigid of the intermediates. Similar changes in the flexibility during the reaction cycle were also assumed from the H/D exchange of kinetics intermediates [95]. These findings indicate that the structural flexibility changes significantly during the catalytic cycle. This is consistent with a movie constructed by Sawaya and Kraut [8] showing that the loops move actively and cooperatively to accommodate both the coenzyme and substrate. The ligand binding caused slight changes (at most 6%) in the solvent-accessible surface area but large changes in the total cavity volume (up to 40%) of ecDHFR. The number, size, and distribution of cavities were correlated with the changes in β_s^o (**Figure 5**). These results suggest that the changes in the flexibilities of the intermediates are dominantly attributable to changes in the cavities [78,79].

Neutron scattering experiment [96] and normal mode analysis [97] showed a softening of vibrational dynamics of ecDHFR on binding MTX to DHFR·NADPH. This result indicates an enhancement of the degrees of freedom responsible for volumetric changes of the protein, consistent with increased β_s^o of the system. It is noticeable that the most significant softening of vibrational dynamics is found in the loops of the protein containing the residues Gly67, Gly121, and Ala145, which have the large effects on stability and function on site-directed mutagenesis [97].

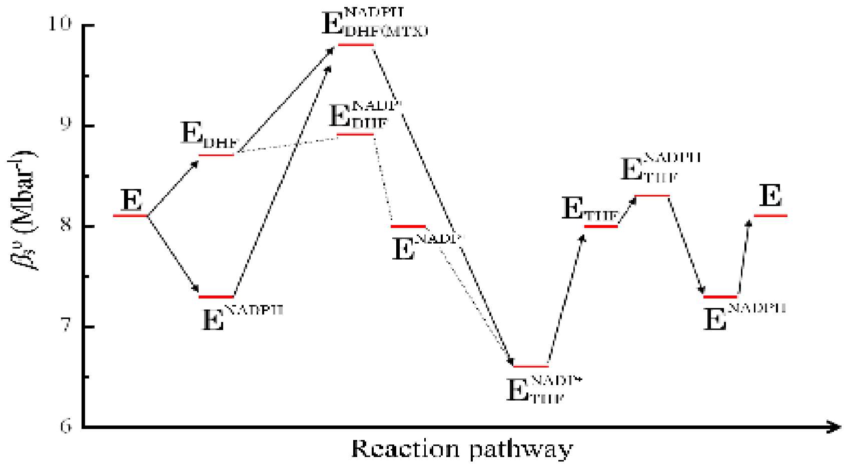

Figure 4: Changes in β_s^o of the kinetics intermediates of ecDHFR in the reaction pathway. MTX refers to methotrexate and other abbreviated letters are the same as in **Figure 2**. The β_s^o value of the transient state DHFR·NADPH·DHF was assumed to be the same as that of the ternary complex DHFR·NADPH·MTX. (Reproduced from Kamiyama and Gekko [78]).

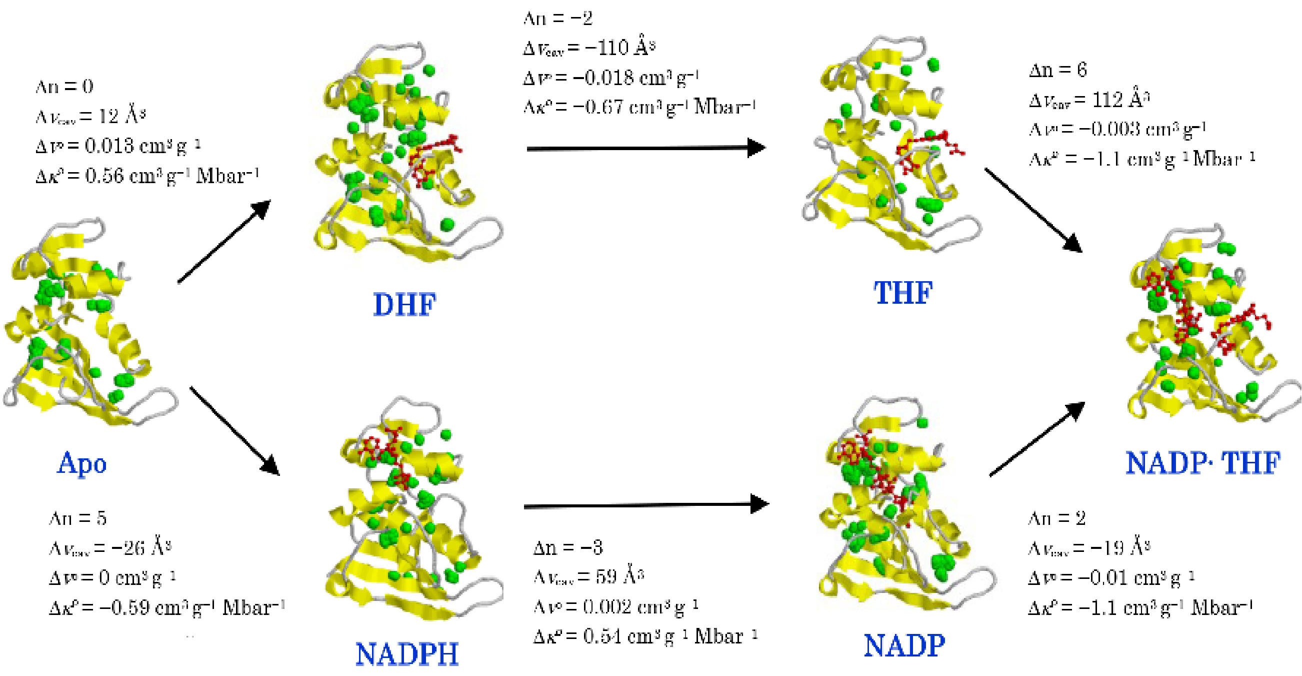

Figure 5: Changes in the number of cavities (Δn), cavity volume (Δv_{cav}), partial specific volume (Δv^o), and adiabatic compressibility [$\Delta\kappa^o = \Delta(v^o\beta_s^{\,o})$] of ecDHFR due to ligand binding. Cavities are shown by green balls. (Reproduced from Kamiyama and Gekko [78].)

Figure 6 shows plots of the K_m and k_{cat}/K_m values against $\beta_s^{\,o}$ for loop substitution mutants. Although the correlations are not significant, $\beta_s^{\,o}$ appears to be negatively correlated with K_m (r = –0.632) and positively correlated with k_{cat}/K_m (r = 0.552), mainly due to the positive correlation between k_{cat} and $\beta_s^{\,o}$ (r = 0.41), with the exception of three mutants (G67T, G67L, and G121V) associated with large experimental errors in $\beta_s^{\,o}$ (>1 Mbar^{-1}). This correlation suggests that the structural flexibility contributes favorably to the enzyme function by enhancing substrate binding and product release, with the latter mechanism dominating. This is supported by a very large $\beta_s^{\,o}$ value observed for a hyperactive ecDHFR mutant along with k_{cat} being sevenfold higher than for the wild type, in which all five methionine and two cysteine residues were replaced by other amino acid residues using a molecular evolutional technique [98]. A similar positive correlation between $\beta_s^{\,o}$ and enzyme function was found for mutants of *E. coli* aspartate aminotransferase at Val39, which is located at the gate of a substrate-binding site [76]. Mutations at residues distant from DNA and cyclic adenosine monophosphate (cAMP)-binding sites of *E. coli* cAMP-receptor protein also induced large changes in $\beta_s^{\,o}$ that were very clearly correlated with the free energy of DNA binding (r = 0.935) and the difference in free energy of binding of two cAMP molecules (r = –0.980) [99]. This demonstrates that the structural flexibility plays an essential role in modulating the DNA binding and the allosteric behavior of the protein.

Together with the mutation effects on $\beta_s^{\,o}$ of the proteins examined so far, a one-unit increase in $\beta_s^{\,o}$ is assumed to enhance the function of the protein by about tenfold. This suggests that the overall fluctuation of protein molecule, being constructed by a complicated mo-

tional hierarchy, is significant in protein function. Although β_s^o does not reveal microscopic features of the structural fluctuation, it should be emphasized that the local structural changes due to loop mutations are dramatically magnified in the overall protein dynamics through the modified cavities so as to affect the enzyme function. Such viewpoint when introduced into experimental and theoretical studies might lead to a deeper understanding of the cooperative interactions between distal residues and the motional network around the active site.

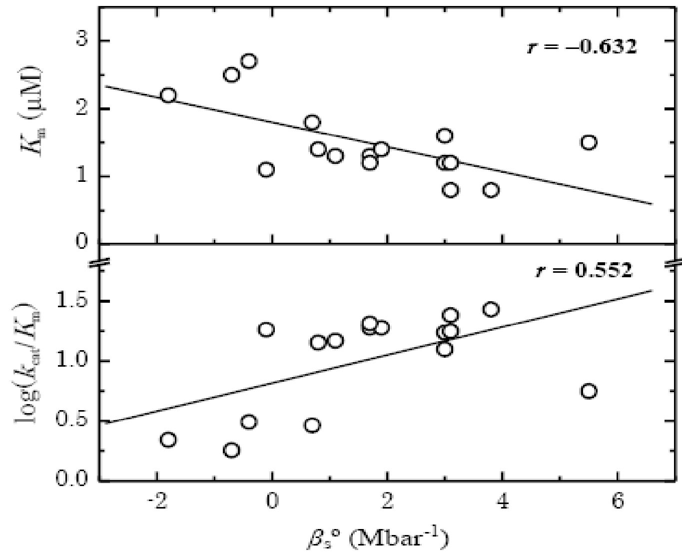

Figure 6: Plots of K_m and log (k_{cat}/K_m) against β_s^o for site-directed substitution mutants at flexible loops of ecDHFR. Solid lines are least-squares linear regressions. (Reproduced from Gekko et al. [77].)

3. DHFRs from Deep-Sea Bacteria

The deep sea is an extreme environment characterized by high hydrostatic pressure up to 110 MPa (a depth of 11,000 m). There live many microorganisms or bacteria depending on the depth: piezophilic, piezosensitive, and piezotolerant species. The pressure adaptation of these deep-sea bacteria has been mainly studied in terms of gene regulation [100–102], but there are limited information at the protein level because the characterization of deep-sea proteins is experimentally difficult. DHFR is a good target enzyme for studying the pressure-adaptation mechanisms of deep-sea proteins because it is an essential enzyme in all the living cell.

As found for ecDHFR, the β_s^o value changes with the kinetics pathway and the enzymatic activity of mutants [77,78,98]. A high-pressure NMR analysis revealed the existence of a pressure-dependent open conformer that would be crucial for NADPH binding [75]. These results suggest that DHFRs from deep-sea bacteria exhibit unique pressure susceptibility and structural dynamics different from those of organisms living under atmospheric pressure. Although the enzymatic activity of ecDHFR decreased with increasing pressure [103], DHFR from *Shewanella violacea* strain DSS12 (svDHFR) isolated from the Ryukyu Trench at a depth of 5,110 m [104,105] was found to exhibit optimal activity at approximately 100 MPa [106]. Therefore, comparative studies of DHFRs from deep-sea and atmospheric-pressure bacteria

should yield useful information for understanding the flexibility–function relationship and the molecular or evolutionary adaptation mechanism of this enzyme to high-pressure environments.

We cloned 17 DHFRs from 11 piezophilic, 5 piezosensitive, and 1 piezotolerant bacterial species isolated from various environments, as listed in **Table 2** [37–40]. The pressure effects on the stabilities and enzymatic activities of 10 of these DHFRs are considered in terms of the internal cavities and surface hydration compared with those of ecDHFR in order to elucidate the species-dependent pressure susceptibility and structural flexibility.

Table 2: DHFR names and original bacterial species described in this study[a].

DHFR name	Bacterial species	Isolation source or depth	Piezophilicity
sb43992DHFR	*S. benthica* ATCC43992	4,575 m	Piezophilic
sb21DHFR	*S. benthica* DB21MT-2	10,898 m	Piezophilic
sb6705DHFR	*S. benthica* DB6705	6,356 m	Piezophilic
sfDHFR	*S. frigidimarina* ACAM591	Antarctic sea ice	Piezosensitive
sgDHFR	*S. gelidimarina* ACAM456	Antarctic sea ice	Piezotolerant
soDHFR	*S. oneidensis* MR-1	Oneida Lake	Piezosensitive
spDHFR	*S. putrefaciens* IAM12079	rancid butter	Piezosensitive
svDHFR	*S. violacea* DSS12	5,110 m	Piezophilic
maDHFR	*M. abyssi* 2693	2,815 m	Piezophilic
mjDHFR	*M. japonica* DSK1	6,356 m	Piezophilic
mmDHFR	*M. marina*	seawater	Piezosensitive
mpDHFR	*M. profunda* 2674	2,815 m	Piezophilic
myDHFR	*M. yayanosii* DB21MT-5	10,898 m	Piezophilic
ppDHFR	*P. phosphoreum*	seawater	Piezosensitive
ppr4DHFR	*P. profundum* DSJ4	5,110 m	Piezophilic
ppr9DHFR	*P. profundum* SS9	2,551 m	Piezophilic
pkDHFR	*Psychromonas kaikoae* JT7304	7,434 m	Piezophilic
ecDHFR	*E. coli*		Piezosensitive

[a]Taken from Ohmae et al. [40].

3.1. Primary and tertiary structures of deep-sea DHFRs

Figure 7 shows the primary structures of deep-sea DHFRs and their normal homologs from congeneric species living in an atmospheric-pressure environment (the nomenclature of each DHFR is listed in **Table 2**). These DHFRs consist of approximately 160 amino acid residues independent of species. The amino acid sequence is considerably conserved at the N-terminal region but highly variable at the C-terminal region. The active-site residue, which is Asp27 in ecDHFR, is completely conserved as Asp28 or Glu28 in all DHFRs. These results suggest that all of these DHFRs adopt a similar folded structure to maintain the catalytic func-

tion.

We determined the crystal structure of a deep-sea DHFR from *M. profunda* (mpDHFR, PDB: 2zza) [39]. The backbone structures of ecDHFR and mpDHFR almost overlap, although their sequence similarity is only 55% (**Figure 7**). Conservation of the backbone structure was also observed in other deep-sea enzymes such as 3-isopropylmalate dehydrogenase (IPMDH) from *S. benthica* strain DB21MT-2 [107], aspartate carbamoyltransferase from *M. profunda* [108], α-glucosidase from *Geobacillus* sp. strain HTA-462 [109], Cu/Zn superoxide dismutase from the deep-sea yeast *Cryptococcus liquefaciens* strain N6 [110], and superoxide dismutase from the deep-sea worm *Alvinella pompejana* [111]. Homology modeling also suggests that deep-sea enzymes have the same folded structures as their normal homologs [112–115]. Human and mouse DHFRs have a similar backbone structure to ecDHFR [116,117] although the sequence similarity is very low (29%), which is comparable to that of 32% for mjDHFR (from *M. japonica* strain DSK1). Considering these results, other deep-sea DHFRs with unknown X-ray structures are expected to have essentially the similar tertiary structures as that of ecDHFR, although their sequence similarity is not particularly high (48–56% in most cases).

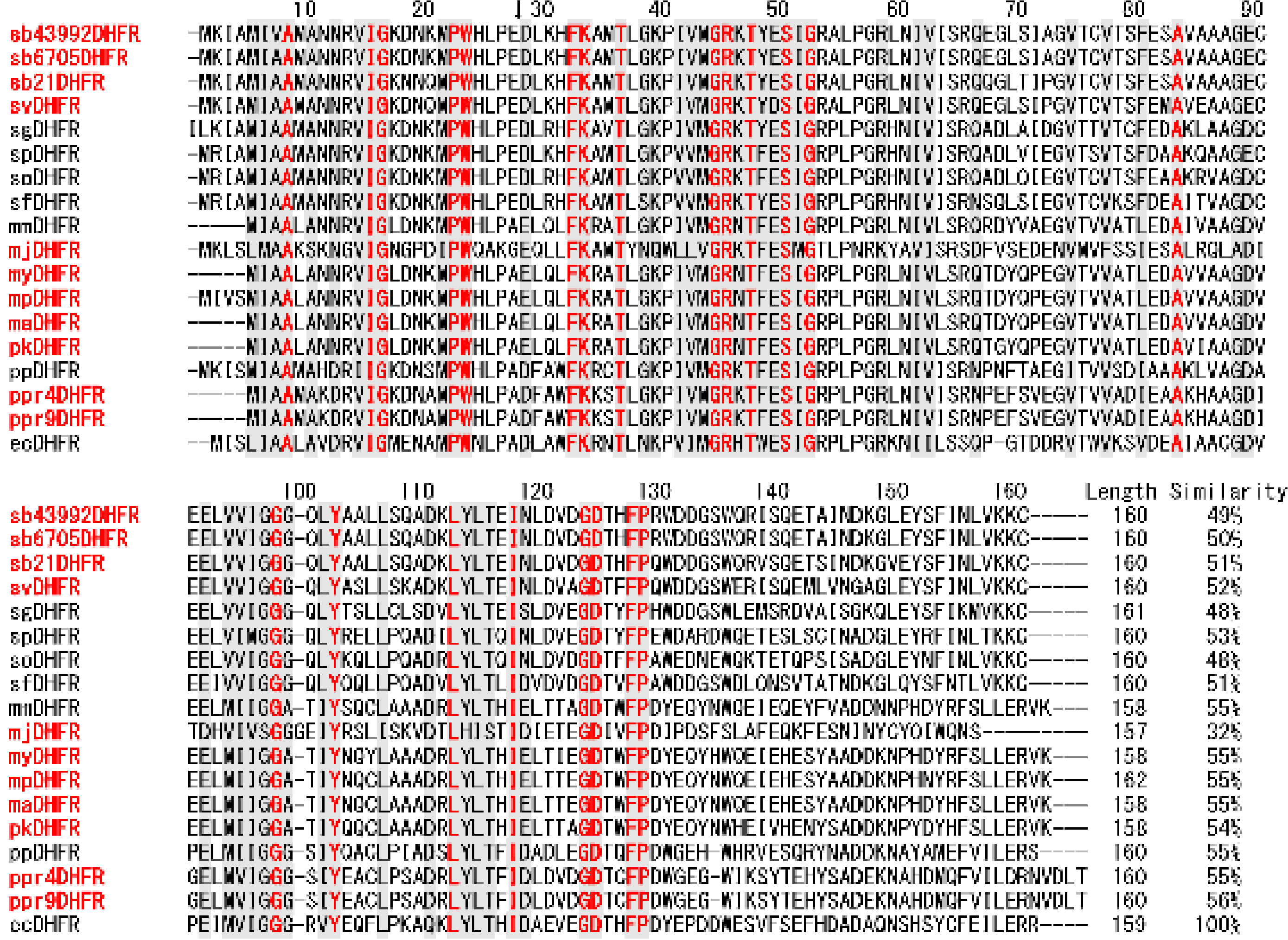

Figure 7: Amino acid sequences of DHFRs from deep-sea bacteria and their congeneric species. Deep-sea DHFRs are indicated by red boldface letters. Residue numbering is based on the sequence of DHFR from *S. benthica*. Conserved amino acid residues are indicated by shading, with fully conserved residues indicated by red boldface letters. The active-site residue is indicated by the arrow on the numbering row. The sequence length and similarity with ecDHFR are also indicated at the end of each sequence. (Reproduced from Ohmae et al. [40].)

Table 3: Thermodynamic parameters for urea denaturation ($\Delta G°_u$, C_m, and m) and steady-state kinetics parameters (K_m and k_{cat}) of deep-sea and atmospheric-pressure DHFRs [a].

DHFR	$\Delta G°_u$ (kJ mol^{-1})	C_m (M)	m (kJ mol^{-1} M^{-1})	K_m (μM)	k_{cat} (s^{-1})
ecDHFR	26.9±3.7 (21.8±1.8) [b]	2.9±0.5 (2.7±0.3)	9.4±1.2 (8.2±0.7)	1.1±0.1	18.4±0.2
sb21DHFR	8.7±1.2	1.9±0.3	4.6±0.3	1.7±0.1	62.7±1.2
sb6705DHFR	7.9±0.9	1.7±0.2	4.7±0.3	2.0±0.2	79.1±1.6
sfDHFR	8.3±0.9	2.1±0.2	4.0±0.2	1.4±0.1	90.7±1.6
soDHFR	6.7±1.0	1.1±0.2	5.9±0.3	1.0±0.1	45.5±0.8
spDHFR	8.3±1.4	1.2±0.2	6.9±0.6	2.4±0.3	96.2±2.9
svDHFR	8.0±0.5	2.3±0.2	3.5±0.2	1.9±0.2	90.1±2.9
mjDHFR	ND	ND	ND	238±26	144.5±3.2
myDHFR	ND	ND	ND	1.8±0.2	83.8±2.4
ppr9DHFR	ND	ND	ND	6.2±0.8	156.0±5.6
mpDHFR	(7.9±0.6)	(1.8±0.2)	(4.3±0.2)		

[a]Thermodynamic and kinetics parameters were determined at 15°C and 25°C, respectively. Taken from Murakami et al. [37,38]. [b] Values in parentheses were determined at 25°C. Taken from Ohmae et al. [39].

3.2. Stability of deep-sea DHFRs

The $\Delta G°_u$, m, and C_m values for urea denaturation of deep-sea DHFRs and their normal homologs are listed in **Table 3** [37–39]. The $\Delta G°_u$ and C_m values of mpDHFR and six *Shewanella* DHFRs were considerably smaller than those of ecDHFR, indicating that these seven DHFRs are less stable than ecDHFR against urea. The small difference in $\Delta G°_u$ among the six *Shewanella* DHFRs may be attributed to their high sequence similarity (more than 80%). The significantly reduced m value of these DHFRs indicates a reduction in the cooperativity of unfolding and that the solvent-accessible surface area does not increase markedly upon unfolding, probably because the native structure of these DHFRs is less compact than that of ecDHFR.

The structural stability against pressure was examined for ecDHFR and mpDHFR using fluorescence spectroscopy [39]. **Figure 8** shows the pressure dependence of the center of fluorescence spectral mass of the two DHFRs at pH 8.0 and various temperatures. It is evident that the transition of mpDHFR shifted to a lower pressure with less cooperativity compared with ecDHFR. The Gibbs free-energy change due to pressure denaturation at atmospheric pressure (0.1 MPa), $\Delta G°_P$, was estimated by extrapolating the ΔG_P values at a given pressure P to 0 MPa:

$$\Delta G_P = \Delta G°_P + P\Delta V° \quad (5)$$

where $\Delta V°$ is the change in partial molar volume due to pressure denaturation at atmospheric pressure. The $\Delta G°_P$ values in the temperature region examined (15.2–28.8°C) were

16.5–21.5 and 2.9–3.3 kJ mol^{-1} for ecDHFR and mpDHFR, respectively, indicating that mpDHFR is more unstable against pressure than is ecDHFR, as also found for urea denaturation [39]. The $\Delta V°$ value at 20.4°C was smaller for mpDHFR (–49 cm^3 mol^{-1}) than for ecDHFR(–74 cm^3 mol^{-1}). The volume change due to urea denaturation was also smaller for mpDHFR (–53 cm^3 mol^{-1}) than for ecDHFR (–85 cm^3 mol^{-1}) at 25°C [39]. Since a negative volume change can be attributed to decreased cavities and/or increased hydration upon denaturation (Eq. 3) and the denatured state should be fully solvated in both DHFRs, the smaller volume changes observed for mpDHFR could be attributed to its native structure being more loosely packed and largely hydrated than that of ecDHFR. This is consistent with the significant pressure and urea-concentration dependences of the fluorescence spectra of native mpDHFR: the solvent molecules are highly accessible to the tryptophan side chains in the interior of the mpDHFR molecule.

The thermal stability of mpDHFR is mysterious. In general, thermal denaturation of a protein as well as other types of denaturation accompany a decrease in the amount of secondary structures. This is the case for ecDHFR, but the molar ellipticity of mpDHFR at 222 nm became more negative with increasing temperature, suggesting an increase in secondary structures upon thermal denaturation [39]. Similar abnormal temperature dependences of CD spectra have been observed for other deep-sea DHFRs (svDHFR and ppr9DHFR; the latter is from *Photobacterium profundum* strain SS9), although these DHFRs aggregated upon thermal denaturation [40]. It is known that the v^o and β_s^o values of native ecDHFR increase markedly with temperature and that its thermal expansion coefficient is two- or threefold higher than those of other proteins [118]. It is therefore possible that deep-sea DHFRs are more flexible at atmospheric pressure. It is unknown whether the thermal stability of deep-sea DHFRs is related to the environmental temperature of the organisms. Further detailed studies of the thermal and pressure denaturation of deep-sea enzymes are necessary, because the effects of pressure and temperature on protein structures are nonadditive, as typically shown by the elliptic *P–T* diagram [79,119–121].

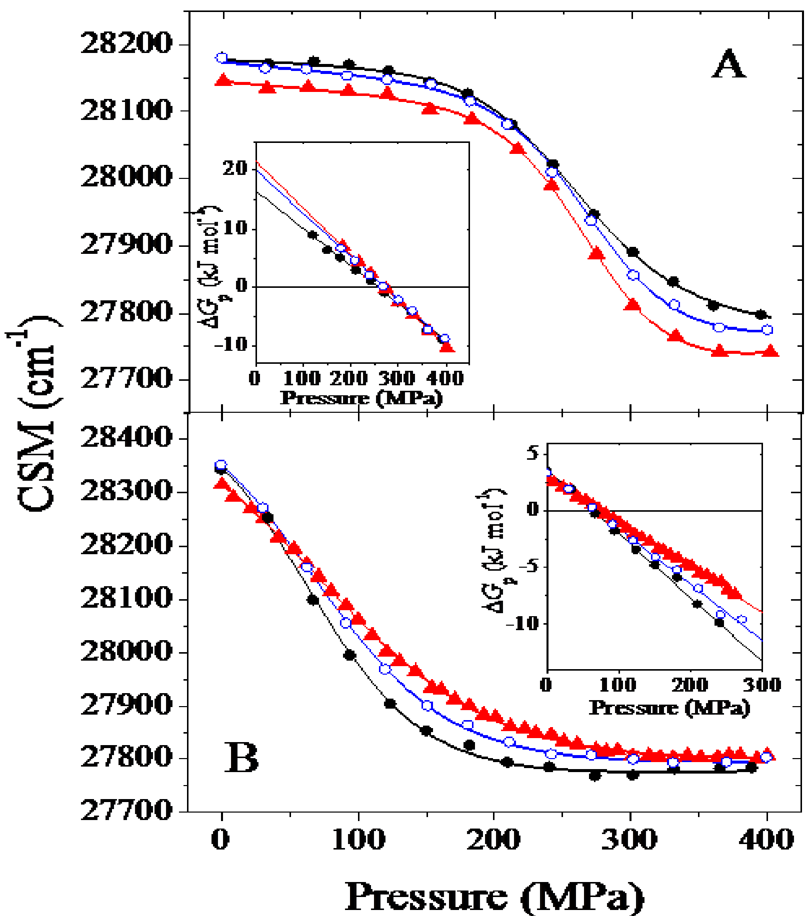

Figure 8: Pressure dependence of the center of fluorescence spectral mass (CSM) of ecDHFR (A) and mpDHFR (B) at pH 8.0. The temperatures were 15.2°C (black filled circles), 20.4°C (blue open circles), and 27.0°C (red filled triangles) for panel A, and 15.7°C (black filled circles), 20.4°C (blue open circles), and 28.8°C (red filled triangles) for panel B. Solid lines represent the theoretical fits to a two-state unfolding model. The insets show the pressure dependence of the apparent Gibbs free-energy change due to pressure denaturation (ΔG_p). (Reproduced from Ohmae et al. [39].)

3.3. Function of deep-sea DHFRs

The K_m and k_{cat} values at atmospheric pressure for ecDHFR, deep-sea DHFRs, and some atmospheric *Shewanella* DHFRs are listed in **Table 3** [37,38]. The K_m values of all of these deep-sea and *Shewanella* DHFRs are larger than that of ecDHFR, especially for mjDHFR. The k_{cat} values of all of these DHFRs are also larger than that of ecDHFR, resulting in a 1.5- to 2.8-fold increase in k_{cat}/K_m with an exceptionally large decrease (28-fold) for mjDHFR. Increases in K_m and k_{cat} were also observed for NADPH, indicating the decreased affinity to a cofactor as well as a substrate [37,38]. Thus, these deep-sea DHFRs are functionally more active than ecDHFR at atmospheric pressure due to the large turnover overcoming the reduced affinity for the substrate, while mjDHFR is less active due to the large reduction in the substrate-binding ability despite the increased turnover.

The effects of pressure on catalytic function are of grave concern for deep-sea enzymes. **Figure 9** shows the pressure dependence of the activities of ecDHFR and some deep-sea DHFRs [38,39]. The activities of three deep-sea DHFRs (svDHFR, mpDHFR, and sb21DHFR; the last is from obligately piezophilic bacterium *S. benthica* strain DB21MT-2 isolated

from the Mariana Trench at a depth of 10,898 m [122]) increased as the pressure increased up to 50 MPa, and then gradually decreased for higher pressures. However, all of the other DHFRs examined did not exhibit such pressure activation, with their activity decreasing monotonically with pressure. This means that deep-sea DHFRs are not necessarily tolerant against pressure, but that some tolerant mechanisms must be involved in these three DHFRs.

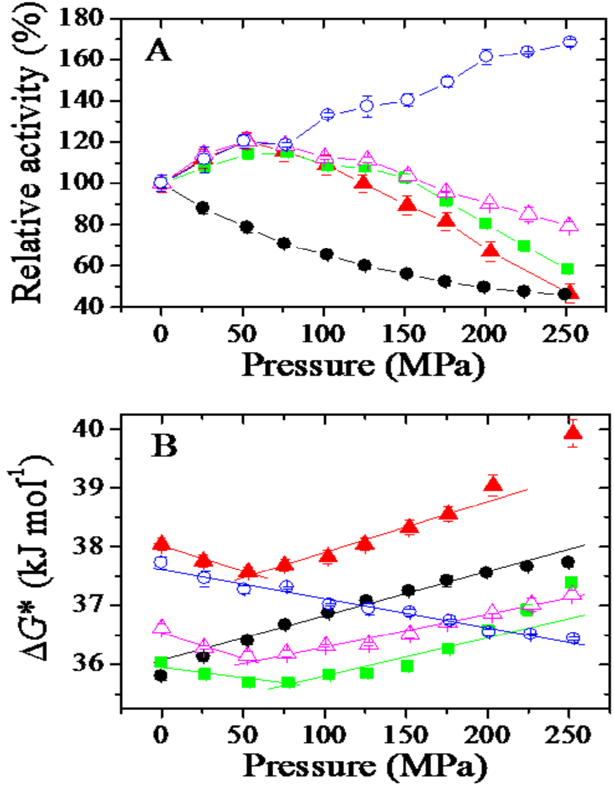

Figure 9: Pressure dependence of the relative activity (A) and activation free energy (B) of deep-sea DHFRs. Three deep-sea DHFRs—mpDHFR (red filled triangles), svDHFR (magenta open triangles), and sb21DHFR (green filled squares)—are compared with wild-type ecDHFR (black filled circles) and its D27E mutant (blue open circles). The experimental temperature was 25.0°C. Lines in panel B indicate linear fits. (Reproduced from Ohmae et al. [40].)

Volumetric data are indispensable for understanding pressure-dependent enzyme reactions. The activation volume (ΔV^*) of a catalytic reaction at a saturated substrate concentration can be estimated from the pressure dependence of the initial velocity (u) of the enzymatic reaction as follows [103]:

$$\Delta V^* = \partial\Delta G^*/\partial P = \partial(-RT\ln(k_{cat}))/\partial P = \partial(-RT\ln(u))/\partial P \quad (6)$$

where ΔG^* is the activation free energy of the enzymatic reaction and ΔV^* is the volume difference between the transition and ground states in the rate-determining step. As shown in **Figure 2**, the enzymatic reaction of DHFR includes at least five steps: two binding steps involving DHF and NADPH, the chemical oxidation–reduction step (hydride transfer), and two releasing steps involving THF and $NADP^+$ [5]. The THF-releasing step is the rate-determining step for ecDHFR at neutral pH and atmospheric pressure. Since the two binding steps can hardly be the rate-determining step at saturated concentrations of DHF and NADPH, ΔV^* would mainly arise from the hydride transfer and the two releasing steps.

The ΔV^* values for the deep-sea and homologous DHFRs are listed in **Table 4**. Three DHFRs (ecDHFR, mjDHFR, and ppr9DHFR) whose activities decreased monotonically with pressure show positive ΔV^* values over the pressure range examined. However, as expected from the inversion in the pressure–activity profile (**Figure 9**), other DHFRs show clearly different ΔV^* values below and above a given pressure (25–125 MPa): ΔV^* changes from negative to positive values at around 50 MPa for svDHFR, mpDHFR, and sb21DHFR. The ΔV^* values for any DHFRs are considerably smaller than the volume change due to pressure denaturation ($\Delta V°$), suggesting that ΔV^* would result from local changes in cavities and hydration around the active site. The positive ΔV^* value may be attributed to dehydration induced by the conformational closing of the transition state upon releasing the product or cofactor, since the hydride transfer step (if it is the rate-determining step) would contribute negatively to ΔV^* through hydration of partial charges or condensation of the hydrated water in the transition state [123,124]. The ΔV^* values in high-pressure regions, which were 5.6, 6.5, and 8.6 $cm^3\ mol^{-1}$ for svDHFR, sb21DHFR, and mpDHFR, respectively, were comparable to that of ecDHFR (7.5 $cm^3\ mol^{-1}$), suggesting that the rate-determining step of these three DHFRs in high-pressure regions is the release of product as well as ecDHFR. Negative ΔV^* values in a low-pressure region would be possible if the rate-determining step changes to the hydride transfer step and/or if the transition state has the open conformation, because both events could accompany the increase in hydration of the transition state.

These interpretations for the ΔV^* values are clearly oversimplified, and so a more-detailed analysis of the pressure effects on each reaction step is necessary for understanding the pressure-adaptation mechanism of deep-sea DHFRs. However, it is apparent that structural flexibility involving modified cavities and hydration participates in the structural and functional adaptation of deep-sea DHFRs to high-pressure environments.

Table 4: Activation volumes at 25°C and pH 7.0 for the enzymatic reaction of DHFRs obtained from bacteria living in deep-sea and atmospheric-pressure conditions [a].

DHFR	ΔV^* ($cm^3 mol^{-1}$)	
ecDFHR (wild type)	7.5±0.2 (0.1–250 MPa)	
ecDHFR (D27E mutant)	–4.8±0.1 (0.1–250 MPa)	
sb21DHFR	–3.5±0.6 (0.1–75 MPa)	6.5±0.1 (75–250 MPa)
sb6705DHFR	2.0±0.1 (0.1–25 MPa)	29.0±0.3 (25–250 MPa)
sfDHFR	14.0±0.1 (0.1–125 MPa)	30.5±0.2 (125–250 MPa)
soDHFR	4.1±1.4 (0.1–50 MPa)	13.1±0.2 (50–250 MPa)
spDHFR	11.5±0.2 (0.1–125 MPa)	23.3±1.0 (125–250 MPa)
svDHFR	–8.6±1.9 (0.1–50 MPa)	5.6±0.1 (50–250 MPa)
mjDHFR	38.7±0.3 (0.1–250 MPa)	
mpDHFR	–8.6±2.5 (0.1–50 MPa)	8.6±0.9 (50–250 MPa)
myDHFR	1.7±0.6 (0.1–75 MPa)	16.5±0.6 (75–250 MPa)
ppr9DHFR	13.8±0.4 (0.1–250 MPa)	

[a] Values in parentheses indicate the pressure range used for the calculation. Taken from Murakami et al. [37,38] and Ohmae et al. [39,134].

3.4. Adaptation mechanisms to deep-sea environments

To understand the adaptation of microorganisms to high-pressure environments, numerous deep-sea piezophilic microorganisms have been isolated and studied over the past 20 years [125,126], focusing on their biodiversity [127,128], pressure-regulated gene expression [129, 130], and genome sequences [131,132]. However, only a few studies regarding protein adaptation to the deep sea have been reported [133–138], and the molecular mechanisms underlying how deep-sea enzymes adapt to high-pressure and other extreme environments remain unclear.

The above-mentioned comparative studies of the stability and function of DHFRs from several deep-sea bacteria and atmospheric-pressure species revealed that three deep-sea DHFRs (svDHFR, mpDHFR, and sb21DHFR) exhibited optimal enzyme activity at approximately 50 MPa. However, pressure decreased the activities of other deep-sea DHFRs such as ppr9DHFR, mjDHFR, and sb6705DHFR (from *S. benthica* strain DB6705). On the other hand, soDHFR from *S. oneidensis* strain MR-1 (isolated from Oneida Lake in the USA [139]) clearly showed pressure tolerance in enzymatic activity up to about 100 MPa [38] despite it being an atmospheric-pressure enzyme. These diverse findings indicate that the activity-optimal pressure of DHFRs is not necessarily correlated with the habitat pressure of the parent bacteria.

According to the taxonomic determination of the isolated deep-sea microorganisms performed on the basis of 5S and 16S ribosomal DNA sequences, approximately half of them

are archaea while others are various kinds of bacteria, but all piezophilic bacteria included in the Gamma-proteobacteria subgroup belong to only five genera: *Shewanella, Moritella, Psychromonas, Photobacterium,* and *Colwellia* [130,140]. Since these five genera also comprise species living in atmospheric-pressure environments, these deep-sea bacteria would have adapted to deep-sea conditions (i.e., high pressure and low temperature) after their genera differentiated in atmospheric-pressure environments. Thus, the high-pressure adaptation mechanism of deep-sea DHFRs would be complicated by evolutionary events.

We recently found that the pressure-dependent activity of ecDHFR was inverted by replacing active-site residue Asp27 with glutamic acid: the activity increased with pressure up to 250 MPa, with a negative ΔV^* (**Figure 9**) [134]. This result is particularly interesting because both amino acids are selected as the active-site residue in deep-sea DHFRs depending on their genera (**Figure 7**). Furthermore, it has been recently found that only a single amino acid substitution dramatically affects the pressure adaptation of a deep-sea enzyme. IPMDH from the extreme piezophile *S. benthica* DB21MT-2 (sbIPMDH) was more pressure-tolerant than that from the atmospheric-pressure-adapted *S. oneidensis* (soIPMDH) despite only a single amino acid differing at the backside of the active center: Ser266 in soIPMDH and Ala266 in sbIPMDH [138]. X-ray structural analyses of soIPMDH indicated that three water molecules penetrated into the cleft around Ser266 under high-pressure conditions so as to reduce the flexibility of the wild-type enzyme, while no water molecule was observed in the Ala266 mutant of soIPMDH that exhibits pressure-tolerant activity similar to that of sbIPMDH [138]. This is the first finding that a single amino acid substitution can play an important role in the pressure adaptation of deep-sea enzymes. This illustrates that mutation studies of deep-sea DHFRs would be fruitful for understanding their pressure-adaptation mechanisms.

4. Concluding Remarks

Structural flexibility is essential for enzyme function. Numerous experimental data and computer simulations have revealed the significant contributions of distal residues to the structural dynamics and catalytic mechanism of ecDHFR. The loop-mutation studies of ecDHFR have demonstrated that local structural changes in flexible loops play substantial roles in the stability, flexibility, and catalytic function of this enzyme *via* modified internal cavities—these loops have not previously been recognized as dynamically and functionally significant. The nonadditive effects of double mutations demonstrate that the motional and energetic coupling between the loops in distant positions is propagated to the active site *via* movement of the Met20 loop. However, a detailed context for the role of dynamics in function remains mysterious because of the complicated motional hierarchy or allosteric effect of protein structure. Further experimental and theoretical studies of distal loop mutants will expand our understanding of the roles of structural flexibility in maintaining the stability and activity of this protein.

In contrast to ecDHFR, deep-sea DHFRs showed significantly different pressure effects on the stability and function, as typically detected in the inversed pressure–activity profile. This also constitutes evidence for the important contribution of internal cavities to structural flexibility. Further comparative studies of DHFRs from various deep-sea bacteria should yield new information to facilitate the understanding of the role of structural flexibility in function and pressure adaptation in relation to molecular evolution. Such information will also be useful for identifying strategies for drug design relevant to DHFR.

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