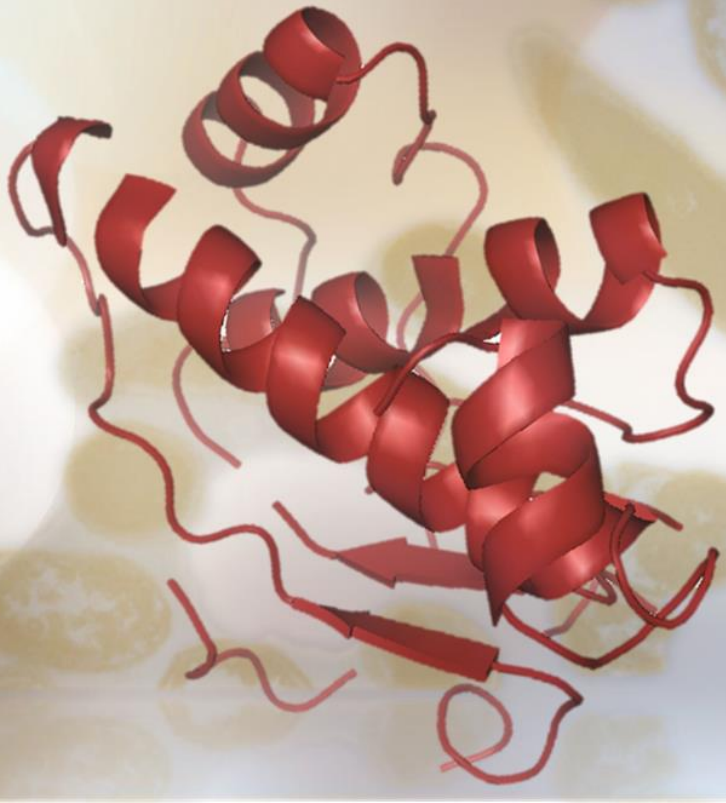
BIOSENSORS AND BIOPROCESSES TO ADDRESS ENVIRONMENTAL POLLUTION

Rosanna Puopolo
Dottorato in Biotecnologie
XXXIII ciclo

Thermophiles



Università di Napoli Federico II

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INDEX

RIASSUNTO Pag. 1

SUMMARY Pag. 6

Chapter [1] - General introduction Pag. 7

Chapter [2] - Metal-Tolerant Thermophiles: From the Analysis of Resistance Mechanisms to their Biotechnological Exploitation. **Review** Pag. 20

Chapter [3] - *Tt*ArsC from *Thermus thermophilus* in biosensors development Pag. 33

- **Paper I**: Self-assembling thermostable chimeras as new platform for arsenic biosensing Pag. 36
- **Paper II**: Phosphatase activity inhibition of the arsenate reductase *Tt*ArsC from *Thermus thermophilus*: its potential application as arsenic biosensor Pag. 48

Chapter [4] - Identification of new heavy metal resistant strains isolated from a hot spring in Italy Pag. 67

- **Paper I**: Identification of a New Heavy-Metal-Resistant Strain of *Geobacillus stearothermophilus* Isolated from a Hydrothermally Active Volcanic Area in Southern Italy Pag. 68
- **Paper II**: Genomic insights of *Alicyclobacillus mali* FL18 isolated from an arsenic-rich hot spring Pag. 83

Chapter [5] - Extremophiles, a nifty tool to face environmental pollution: from exploitation of metabolism to genome engineering. **Review** Pag. 101

Chapter [6] - General conclusion & Acknowledgement Pag. 126

Appendix I - Publications & Author's contribution Pag. 130

Appendix II – Participation to congresses Pag. 132

Appendix III - Experiences in foreign laboratories Pag. 134

RIASSUNTO

L'inquinamento ambientale fin dalla rivoluzione industriale del XIX sec è un fenomeno in continuo aumento. Può essere definita "inquinante" qualunque sostanza, di origine naturale o antropica, che non rientri nella composizione della matrice ambientale di interesse, o che sia in essa presente a concentrazioni nettamente superiori ai valori naturali, provocando un effetto dannoso per l'ambiente e di conseguenza per la salute umana. Combattere l'inquinamento ambientale, e quindi proteggere l'ambiente, è una delle maggiori sfide del mondo contemporaneo, poiché ne coinvolge direttamente il futuro stesso.
I metalli pesanti sono tra le sostanze più tossiche e persistenti presenti nell'ambiente; sebbene non ci sia tutt'ora una definizione universalmente accettata di "metallo pesante", generalmente vengono definiti tali tutti i metalli e metalloidi di alta densità e numero atomico, che possono essere nocivi per la salute dell'uomo e dell'ecosistema, tra cui ferro, cadmio, cobalto, mercurio, piombo e arsenico. Quest'ultimo è stato oggetto di particolare studio in questo progetto di tesi. L'arsenico è uno dei metalloidi più abbondanti della crosta terrestre e delle acque, sia marine che dolci, dove è presente in diversi stadi di ossidazione, sia sotto forme organiche che inorganiche, queste ultime più tossiche e prevalenti, ovvero arseniato As(V) e arsenito As(III).
Arsenico ed altri metalli pesanti sono molto abbondanti in alcune aree del nostro pianeta, come le sorgenti geotermali (marine e terrestri), habitat considerati estremi poiché, oltre ad elevate concentrazioni di metalli, presentano condizioni estreme, quali temperature e/o livelli di pH molto alti o bassi, nonché alte concentrazioni saline. Anche in queste condizioni è presente la vita, infatti vi si sono evoluti diversi microrganismi estremofili (termofili se vivono a temperature superiori ai 50 °C) appartenenti ai domini dei *Bacteria* e degli *Archaea*, che possiedono meccanismi dinamici e versatili per contrastare stress chimici e fisici, e sopravvivere in condizioni ambientali considerate ostili per altre forme di vita. Questi microrganismi hanno dunque sviluppato sistemi metabolici capaci di contrastare la tossicità dei metalli pesanti e pertanto partecipare attivamente alla loro mobilizzazione e conversione chimica. Tali caratteristiche hanno attratto gli scienziati anche da un punto di vista applicativo per lo sviluppo di bioprocessi volti al risanamento ambientale.
Nell'ottica di contrastare l'inquinamento ambientale, un'attività fondamentale è quella del monitoraggio delle sostanze inquinanti. Nel caso dei metalli pesanti, tale obiettivo è ad oggi perseguito prevalentemente attraverso approcci tradizionali che prevedono

l'utilizzo di strumentazioni ad alto costo e specializzazione, come spettrometri di massa e sistemi di cromatografia. Questi strumenti consentono determinazioni estremamente precise e accurate della quantità di metallo presente nei campioni, ma da diversi anni ormai, l'attenzione della comunità scientifica si è concentrato sullo sviluppo e ricerca di strategie più semplici che permettano di fare misure in loco e in tempo reale; tra queste, di particolare interesse sono i biosensori.

I biosensori sono dispositivi che utilizzano un elemento di riconoscimento biologico, detto anche biomediatore, collegato ad un trasduttore di segnale, per la determinazione selettiva di un analita. In particolare, il biomediatore, che può essere costituito da microrganismi, tessuti, organelli, recettori cellulari, enzimi, anticorpi o acidi nucleici, è in grado di interagire con l'analita e viene immobilizzato sulla superficie del sensore; in seguito all'interazione con l'analita si verifica una variazione di un parametro chimico o fisico, che viene rivelata dal trasduttore, in grado di convertirla in un segnale misurabile.

Nello sviluppo di un biosensore, una delle criticità è la resistenza e la stabilità del materiale biologico. Le proteine derivanti da microrganismi termofili sono state ampiamente caratterizzate per resistenza e stabilità, tanto da essere da anni impiegate in diversi settori industriali.

Il presente lavoro di tesi, intitolato "Biosensori e Bioprocessi per combattere l'inquinamento ambientale" verte sullo sfruttamento di microrganismi termofili per applicazioni ambientali, in particolare offre una panoramica su quelli metallo-resistenti, sui loro sistemi di resistenza e sulle loro applicazioni biotecnologiche (**Capitolo [2]**); lo sviluppo di nuovi biosensori per l'arsenico basati su enzimi termofili (**Capitolo [3]**); l'isolamento e la caratterizzazione di nuovi microrganismi termofili metallo-resistenti da ambienti estremi (**Capitolo [4]**) e infine un aggiornamento sulle nuove tecniche di ingegnerizzazione di tali termofili, e sulla loro applicazione in campo ambientale oltre che su metalli pesanti, anche su inquinanti organici e nello smaltimento di biomasse lignocellulosiche (**Capitolo [5]**).

Punto di partenza di questo lavoro di tesi è stata la conoscenza del sistema di resistenza all'arsenico del batterio *Thermus thermophilus* HB27. Si tratta di un batterio termofilo Gram- negativo, che cresce a 70 °C; in particolare possiede un repressore trascrizionale *Tt*SmtB che rispondendo alla presenza di arsenico, regola l'espressione di un'arseniato reduttasi (*Tt*ArsC) capace di convertire As(V) in As(III), di un'ATPasi di membrana (*Tt*ArsX) responsabile dell'efflusso di As(III) dalla cellula, e interagisce con un'arsenito metiltransferasi (*Tt*ArsM) che metila As(III) libero rendendolo meno tossico.

L’arseniato reduttasi *Tt*ArsC è protagonista del Capitolo [3], in cui viene utilizzata per lo sviluppo di potenziali biosensori di tipo elettrochimico e ottico. Nel primo caso, *Tt*ArsC è stata fusa geneticamente con un’idrofobina (Vmh2) del fungo edibile *Pleurotus ostreatus*, una proteina dalle capacità adesive e auto-assemblanti, al fine di ottenere una proteina chimerica che combinasse le caratteristiche enzimatiche di *Tt*ArsC, nonché di resistenza vista la sua natura termofila, e quelle adesive di Vmh2 per una corretta e semplice immobilizzazione del biomediatore. In particolare, sono state prodotte due versioni della chimera: ArsC-Vmh2 e Vmh2-ArsC; la prima presenta all’estremità N-terminale della proteina l’arseniato reduttasi, viceversa la seconda presenta l’idrofobina. Entrambe le proteine chimeriche sono state espresse in maniera eterologa in *Escherichia coli*, e purificate da corpi di inclusione, nello specifico Vmh2-ArsC è sia più espressa, che meglio purificata in termini di resa della sua controparte ArsC-Vmh2. Entrambe le chimere sono state testate sia per la loro attività enzimatica, che per la loro resa di immobilizzazione su superfici di polistirene e d’oro; infine sono state impiegate come biosensori per As(III), in particolare sono state immobilizzate su elettrodi d’oro per la cattura di As(III) in soluzioni acquose, con conseguente rivelazione elettrochimica dovuta alla riduzione di As(III) in As(0) sulla superficie dell’elettrodo. I risultati hanno dimostrato che sia ArsC-Vmh2 che Vmh2-ArsC, possono essere utilizzate nello sviluppo di biosensori elettrochimici per As(III).
Nella seconda parte del capitolo, è stato invece approfondito lo studio di un’attività secondaria di *Tt*ArsC; l’arseniato reduttasi di *T. thermophilus* infatti possiede anche un’attività fosfatasica dovuta alla presenza nella sua struttura tridimensionale di un P-loop, omologo a quello di proteine tirosin-fosfatasi a basso peso molecolare (LMW PTPasi). Si ipotizza che la stessa attività ossido-reduttasica si sia evoluta a partire da quella fosfatasica come conseguenza di una pressione evolutiva dovuta alla comparsa dell’arseniato nell’ambiente e al divenire dell’atmosfera ossidante. Nello specifico di questo lavoro, è stata valutata l’interferenza dell’arsenico come inibitore dell’attività fosfatasica di *Tt*ArsC; come substrato è stato utilizzato il para-nitrofenilfosfato, sostanza che una volta idrolizzata rilascia fosfato inorganico e para-nitrofenolo (pNP), un prodotto giallo con assorbimento massimo a 405 nm. Lo studio delle cinetiche ha rivelato che sia As(V) che As(III) danno luogo a un’inibizione non competitiva dell’attività fosfatasica, ma la quantità di arsenico richiesta per inibire totalmente la reazione è di circa due ordini di grandezza inferiore nel caso di As(V) rispetto ad As(III). Questa specificità per l’As(V), dovuta presumibilmente al fatto che si tratta del substrato naturale di *Tt*ArsC,

è stata valutata anche in presenza e rispetto ad altri metalli pesanti e pesticidi appartenenti alla classe degli organofosfati, nonché in soluzioni saline, al fine di valutare la potenziale applicazione di questo sistema per lo sviluppo di un biosensore. I risultati hanno evidenziato che l'attività fosfatasica di *Tt*ArsC può essere utilizzata per lo sviluppo di biosensori a inibizione per l'As(V), con limiti di rilevabilità in linea con altri enzimi caratterizzati in letteratura allo stesso scopo, e specificità anche superiore.

L'isolamento e la caratterizzazione di nuovi microrganismi termofili metallo-resistenti da ambienti estremi, rappresenta l'altra macroarea sperimentale di questa tesi, e viene affrontata nel Capitolo [4]. In particolare, in due momenti diversi, sono stati isolati due nuovi ceppi di *Geobacillus stearothermophilus* e *Alicyclobacillus mali* dalla stessa sorgente geotermale nell'area solfatarica dei Campi Flegrei (Napoli). Si tratta in entrambi i casi di batteri termofili, il cui optimum di temperatura di crescita è stato determinato rispettivamente a 60 °C (a pH 7) e 55 °C (a pH 4). Mentre per *G. stearothemophilus* sono state effettuate caratterizzazioni fenotipiche e morfologiche, per *A. mali* è stata anche condotta un'analisi genomica, che ha rivelato la presenza di numerosi geni putativi caratteristici dei sistemi di resistenza per l'arsenico. Entrambi i microrganismi hanno mostrato tolleranza verso diversi metalli pesanti, e in particolare verso l'arsenico, con una propensione per l'As(V) da parte del primo, e per l'As(III) da parte del secondo. Degno di nota, *A. mali* si è rivelato resistente anche a numerosi antibiotici; si tratta di un fenomeno raro e ancora poco studiato nei microrganismi termofili isolati da ambienti estremi naturali, che per loro natura hanno subito poca contaminazione da parte dell'uomo. Più in generale, lo studio dei meccanismi di resistenza di questi microrganismi rappresenta il punto di partenza per lo sviluppo di nuovi sistemi biologici e/o bioprocessi per il monitoraggio ambientale e il biorisanamento.

Le peculiarità dei microrganismi termofili e delle loro componenti, rappresentano una risorsa in vari campi applicativi, ma specialmente nel settore industriale e ambientale, dove la versatilità di adattamento per la crescita su scarti e/o inquinanti si configura come uno strumento attraverso il quale, non solo contribuire alla tutela dell'ambiente, ma anche generare prodotti biotecnologici ad alto valore aggiunto. In questo contesto si fa sempre più strada l'esigenza di ingegnerizzare tali microrganismi per ottimizzarne le prestazioni, e in effetti, a tale scopo nuovi strumenti e avanzamenti tecnologici sono stati resi disponibili negli ultimi anni.

I risultati sperimentali ottenuti durante questo percorso di dottorato hanno permesso di ampliare la conoscenza dei microrganismi termofili,

maturata da uno studio bibliografico nell'ambito industriale e ambientale. Nello specifico, la tesi si è sviluppata lungo due macroaree: lo sviluppo di biosensori, in cui sono state messe a punto nuove piattaforme e potenziali strategie per il monitoraggio dell'arsenico, sfruttando l'arseniato reduttasi di *T. thermophilus*; e l'isolamento e la caratterizzazione di nuovi microrganismi termofili potenzialmente utili allo sviluppo di bioprocessi per il risanamento ambientale. I termofili si confermano come microrganismi dalle straordinarie proprietà, estremamente versatili, e potenziali risorse biotecnologiche.

SUMMARY

Pollution caused by heavy metals is a serious threat for the environment and human health, because of their toxicity and persistency. Among heavy metals, arsenic represents a harsh pollutant able to contaminate air, water and soil; it is a component of the Earth crust and is present in many geothermal environments, but it is also released into the environment by the consumption of arsenic-containing products such as insecticides, pesticides, and chemotherapeutic drugs.

Most of the current systems for monitoring and restoring the environment from heavy metals require several expensive and hard instrumentation; modern biotechnologies can be addressed to exploit metal bio-transformations for the set-up of easier and possibly cheaper devices and eco-sustainable processes.

In this context thermophiles stand out, they are microorganisms adapted to live in harsh conditions, which often include high concentrations of heavy metals. Therefore, knowledges of their resistance mechanisms can lead to the development of new strategies to face the heavy metals pollution. Moreover, thermophilic microorganisms have been associated to sources of thermostable proteins and enzymes useful for several applications; in fact, since the last 30 years they have been deeply studied for their high potential in industrial biotechnologies.

This PhD thesis is aimed at the exploitation of thermophilic microorganisms for environmental applications. In particular we here report the state of the art on the most common thermophilic resistance mechanisms to heavy metals (**Chapter [2]**); we also describe the set-up of electrochemical and optical biosensors for the monitoring of arsenic based on thermophilic enzymes (**Chapter [3]**), as well as the isolation and physiological, molecular and genetic characterization of new metal-tolerant thermophiles from extreme environments (**Chapter [4]**) as source of novel biomolecules and/or bioprocesses. Furthermore, we report the emerging technologies for genetic engineering of thermophilic microorganisms, and their employment against, not only heavy metals, but also organic pollutants and in the lignocellulose degradation (**Chapter [5]**).

Chapter [1] - Introduction

Environmental Pollution by Heavy Metals

Addressing the environmental pollution is one of the most serious global challenges of this century. Since the onset of the industrial revolution during the 19th century, this phenomenon has grown up to reach global proportions; in fact environmental pollution is a problem not only in the developed countries but also in developing ones, where legislation on environmental safety is less strict [1]. The nature of pollutants is very heterogeneous: any chemical substance can be defined a pollutant, if it is unexpectedly found in the environment at a concentration higher than the permissible limits [2]. Therefore, monitoring the pollution, but also finding strategies to recover and re-use pollutants are points of interest for biotechnological applications.

Heavy metals are among the most persistent and toxic pollutants. Even in small concentrations, they can threat human health as well as the environment because they are not biodegradable [3]. Exposure to heavy metals can cause several human diseases, such as respiratory problems, kidney pathology, neurological disorders, and cancer: for example, lead provokes kidney problems and rises blood pressure, while arsenic is carcinogenic and causes skin damages and problems to circulatory system [4]. The term “heavy metals” is widely referred to a group of metals and metalloids associated with potential toxicity or ecotoxicity. Generally, these metals possess relatively high densities, atomic weights, or atomic numbers, but the criteria used for this classification can vary depending on the author and the contest [5]; the International Union of Pure and Applied Chemistry (IUPAC), gave some guidelines considering as “heavy” every metal presenting the following characteristics: density exceeding 5.0 g/cm^3; general behavior as cation; low solubility of their hydrates; aptitude to form complexes and affinity towards the sulfides [6].

Heavy metals can contaminate air, water, and soil, and subsequently cause health issues in plants, animals, and people. Common sources of them include mining and industrial wastes, vehicle emissions, lead-acid batteries, fertilizers, paints, and treated timber, aging water supply infrastructure, and microplastics floating in the world's oceans [7,8]. Despite their relevant toxicity, their use is intended to extend over time, since many heavy metals, like copper or nickel, have been identified by the European Commission as critical raw materials for the transition to green energy technologies [9,10]. In fact, according to the sustainable low-carbon economic policy of EU, such metals could become a

bottleneck to the supply-chain of various low-carbon energy technologies [11].

Arsenic

Arsenic is one of the most abundant metalloids of the Earth's crust. As other natural inorganic elements, arsenic is released by natural processes such as weathering of rocks, volcanic eruptions, and geothermal waters. However, anthropogenic activities from industrial and agricultural fields contribute to contaminate the environment by arsenic: examples are the burning of fossil fuels, the mining activities, or the use of arsenic-based herbicides, fungicides and pesticides [12]. At high concentrations arsenic is very toxic and dangerous for human health. More than 100 million people in the world are at risk from consuming water contaminated by arsenic, since it can contaminate both groundwater sources and soils [13]. Therefore, strategies to detect and prevent this global problem are urgently required.

Inorganic arsenic can be found in four different oxidation states: arsenite As(III) and arsenate As(V) are the most common (Figure [1.1]), while the elemental arsenic As(0) and arsenide As(-3) are more rare [14]. Moreover, it is accumulated in sediments, water and soil either in inorganic forms, with the prevailing of the pentavalent or trivalent arsenic respectively under oxidizing or reducing conditions, or in organic form as the mono-methylarsonic acid (MMA) and di-methylarsinic acid (DMA) [15]. Organic arsenic forms are less toxic than the inorganic ones, because they can permeate through the cell membrane. As(V) thanks to its structural similarity with the phosphate ion, inhibits the oxidative phosphorylation; while As(III) binds the thiol groups of proteins, causing an irreversible inhibition [14,16]. In fact, the arsenic methylation was previously regarded as a detoxification pathway, but recent studies have suggested that the main intermediate metabolites MMA and DMA are reactive and toxic in themselves [17]. Nevertheless, since arsenic has different valence states, its treatment and removal from the contaminated sites is not an easy issue.

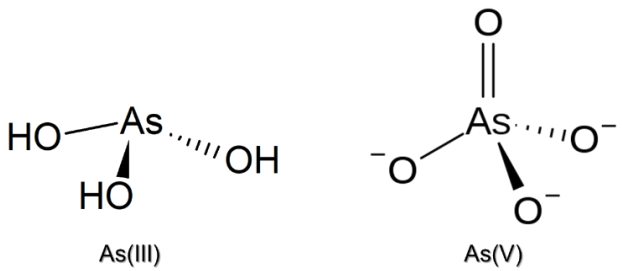

Figure [1.1] Structures of the most common forms of inorganic arsenic: As(III) and As(V).

Biosensors & Bioprocesses

Traditional approaches for monitoring environmental pollutants are based on chemical or physical analysis and allows highly accurate and sensitive determination of the exact composition of any sample. However, these analyses require specialized and expensive instrumentations, such as chromatographic systems and/or mass spectrometers. The need for accurate, less expensive and on-site measurements has led to the development of sensors based on biomolecules: biosensors [18,19].

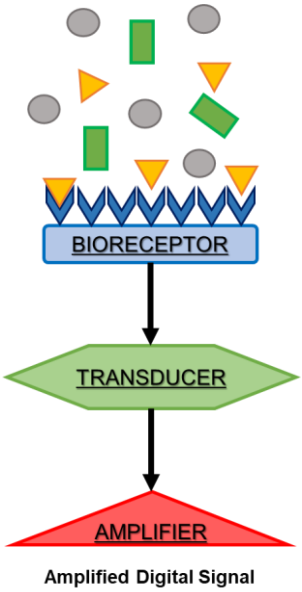

Figure [1.2] Schematic representation of a biosensor.

A biosensor is an analytical device which integrates a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of an analyte [20–22] (**Figure [1.2]**).

The major advantage of using biological molecules is their high specificity: biosensors can be based on the specific interactions between enzymes and their substrates, the recognition between antibodies and antigens, accessibility of specific target molecules to their receptors, or the high affinity of nucleic acid strands to their complementary sequences [20]. On the other hand, obstacles to a larger employment of biosensors are fundamentally related to the "weakness" of biomaterials, and to their immobilization on biosensor surfaces. To solve this last issue, particular attention is payed to self-assembling proteins; an example is represented by hydrophobins

(amphipathic, highly surface-active, and self-assembling fungal proteins) which form amyloid fibrils and are promising candidates for the fabrication of functional materials and biosensors [23,24].
The even more significant attention to the environment has concentrated some technologies on the transformation of hazardous wastes to produce desired products without waste byproduct, as well as on the remediation of contaminated sites. Many of these technologies are microbial based, therefore they can be defined as *bioprocesses*. A bioprocess, in its broadest sense, is a specific process that uses complete living cells or their components (e.g., bacteria, enzymes, chloroplast) to obtain desired products [25]. Regarding heavy metals, microbe–metal interactions are studied in different branches of metal biotechnology, that have led to the development of biotechnological processes for the extraction of metals from ores (biomining), bioremediation of contaminated sites, treatment of metal-containing wastes and recovery of metals [26].

Metal tolerant thermophiles

Thermophiles are microorganisms adapted to live at high temperatures, from 40 to 70 °C they can be classified as *moderate thermophiles*, while from 70 to 80 °C as *extreme thermophiles*; microorganisms living over 80 °C up to 125 °C (the current upper limit of life) are considered *hyperthermophiles* (**Figure [1.3]**). Many of thermophilic microorganisms belong to both the kingdoms of *Archaea* and *Bacteria*. They are found in several natural habitats, that are geothermally active regions, as hot springs, and deep-sea hydrothermal vents.
Ever since their discovery, thermophiles have been object of considerable interest in biotechnologies; industrial processes involving them have numerous advantages, including reduced risk of contamination, improved solubility of substrates, continuous recovery of volatile chemical products directly from fermentation broth, and reduced cooling costs [27,28]. Moreover thermophilic enzymes, which have their optimal activity at high temperatures, are more stable than the correspondent mesophiles to other harsh conditions, therefore they have been massively employed, starting from the development of the polymerase chain reaction (PCR) [29] to become powerful tools for industrial catalysis [30–33].

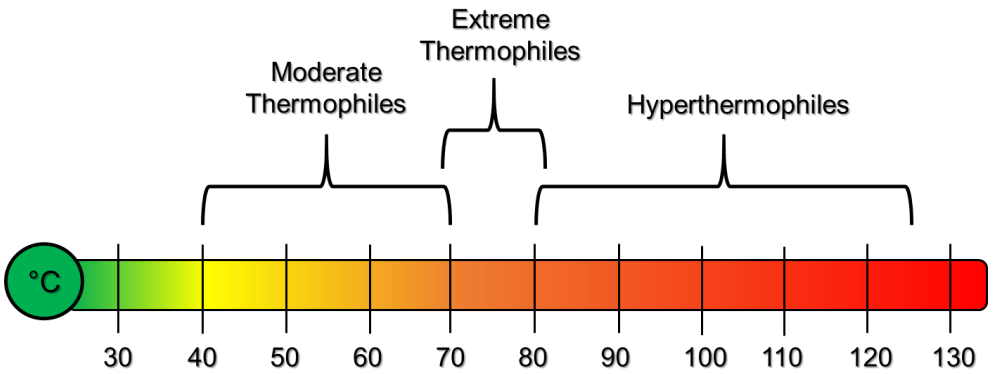

Figure [1.3] Classification of thermophiles.

Microorganisms able to tolerate high levels of heavy metal ions have evolved in ore deposits, hydrothermal vents, geothermal sites, as well as in different polluted sites [27]. In particular, metal-tolerant *thermophiles* exhibit metabolic and physiological features that distinguish them from other major life groups due to their adaptation to survive at high metal concentrations [34]. Metal tolerance of thermophilic Bacteria/Archaea is due to several mechanisms, many also found in their mesophilic counterparts, such as: extracellular barrier, metal ion transport into and outside the cell, utilization of toxic metal ion in metabolism or presence of metal resistance genes with different genomic localization (chromosome, plasmid or transposon) [35]. Understanding the molecular mechanisms responsible for resistance to heavy metals in metal-tolerant *thermophiles* is crucial to exploit them in environmental monitoring or to set up bioremediation processes, intended to contrast the heavy metals pollution.

Arsenic resistance in *Thermus thermophilus*

T. thermophilus is an aerobic Gram-negative thermophilic bacterium, that grows at temperatures ranging from 50 to 82 °C. It has been extensively studied as model organism of thermophiles thanks to its high growth rates, cell yields of the cultures, the availability of genetic tools, and the constitutive expression of an impressively efficient natural competence apparatus [36]. The HB27 strain was originally isolated from a natural thermal environment in Japan by Oshima and coworkers and it grows optimally at 70 °C and at pH 7.0 [37]. Investigations on the molecular mechanisms responsible for *T. thermophilus* HB27 arsenic tolerance, showed resistance towards arsenate and arsenite up to concentrations of 44 mM and 40 mM, respectively. Unlike the majority of microorganisms which encode *ars* operons, the main components of the arsenic detoxification system have been found interspersed in the

chromosome; in particular they code for an arsenate reductase (*Tt*ArsC)[38], a cation-transporting membrane ATPase (*Tt*ArsX)[39] and a transcriptional regulator belonging to the ArsR/SmtB family (*Tt*SmtB) [40,41]. In particular, *Tt*SmtB is an auto repressor and also represses *Tt*ArsC and *Tt*ArsX expression: in absence of arsenic, *Tt*SmtB is expressed at low levels and is bound to its target promoters (*TtsmtB*, *TtarsC,* and *TtarsX*). When As(V) enters into the cell, it interacts with *Tt*SmtB that changes its conformation, becomes less affine to DNA and derepresses *TtsmtB*, *TtarsC* and *TtarsX* transcription (*TtarsX* is more tightly repressed and needs that *Tt*SmtB binds As(III) to be completely derepressed); the increased levels of the arsenate reductase, determine the catalytic conversion of As(V) into As(III). The intracellular accumulation of As(III) causes the complete release of the *TtarsX* promoter, transcriptional activation of the gene and active extrusion of As(III) outside the cells (**Figure [1.4]**) [40]. Moreover, a functional characterization of this system revealed a common/promiscuous mechanism of defense from both arsenic and cadmium [39]. Furthermore, recently our group discovered that *Tt*SmtB interacts and regulates the expression of another component of the arsenic resistance system, an arsenite-methyltransferase (*Tt*ArsM). We showed that the enzyme has methyltransferase activity, and the detailed characterization of the enzyme products and catalytic mechanism is underway [42].

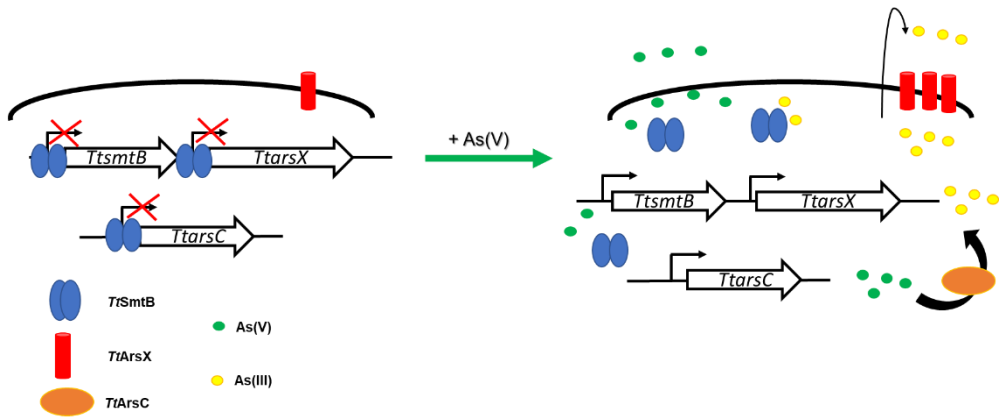

Figure [1.4] Schematic representation of the arsenic resistance system in *T. thermophilus*. As(V) enters the cell and binds *Tt*SmtB lowering its affinity towards DNA and inducing the de-repression of *Tt*smtB and *Tt*arsC promoters; the expressed *Tt*ArsC reduces As(V) into As(III). Once As(III) is accumulated, it binds *Tt*SmtB determining the complete release of the *Tt*asrX promoter, and therefore the transcriptional activation of the pump which extrudes As(III) outside the cell.

The elucidation of the molecular mechanisms responsible of arsenic resistance system has already allowed the exploitation of *T. thermophilus* HB27 and its components in the set-up of different biomonitoring systems: in particular, the microbe has been engineered (at plasmidic and genomic levels) to realize a whole-cell biosensor for As(III), As(V) and Cd(II) [39]; and its arsenate reductase, *Tt*ArsC, has been adsorbed to polyethylene glycol-stabilized gold nanospheres to obtain an optical nanobiosystem for arsenic sensing [43,44]. *T. thermophilus* and its molecular determinants have demonstrated to be good candidate for heavy metals biosensing applications; however scientific research never stops and pushes to find solutions that are really applicable on the market, outside a lab scale [45]. Therefore, it is always important to find new strategies, and improve the results obtained.

Aim of the thesis

The work described in this thesis has been focused on a) the development of biotechnological systems for the monitoring of arsenic, and b) on the isolation and characterization of new thermophilic microorganisms tolerant to heavy metals.
The first objective has been achieved thanks to the previous knowledge of the molecular determinants of the arsenic resistance system in *T. thermophilus*, while the second one was pursued with the aim of expanding the set of systems usable for environmental biomonitoring and/or bioprocesses.

The **Chapter [2]** contains an overview of the main heavy metal microbial resistance systems in thermophilic microorganisms, and their biotechnological applications.
The **Chapter [3]** is focused on the exploitation of *Tt*ArsC, the arsenate reductase of *Thermus thermophilus* for the development of new enzyme-based arsenic biosensors. In Paper I it was genetically linked to the hydrophobin Vmh2, and As(III) measured with a novel electrochemical detection. In Paper II, it is described the realization of an As(V) detection system based on the inhibition of *Tt*ArsC phosphatase activity by As(V).
The **Chapter [4]** describes the characterization of new thermophilic metal tolerant microorganisms isolated from a hot spring located in a solfataric area of Pozzuoli (Naples, IT). Paper I reports the characterization of *Geobacillus stearothermophilus*, and Paper II that of *Alicyclobacillus mali*.

The **Chapter [5]**, finally, is a review article describing the current knowledge on the biotechnological applications that exploit extremophiles for environmental purposes, including available tools for manipulative genetics of thermophiles.

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Chapter [2]: Metal-Tolerant Thermophiles

From the Analysis of Resistance Mechanisms to their Biotechnological Exploitation

Extremophilic microorganisms are adapted to survive in extreme environments such as high or low temperatures, high pressure, high salt concentrations and extreme pHs. These microorganisms are a biotechnological treasure for many bioprocesses because they produce unique biocatalysts that work under conditions comparable to those prevailing in various industrial processes.
Moreover, studies on extremophilic organisms significantly contribute to the comprehension of the molecular mechanisms responsible for either adaptation to extreme conditions and response to different chemical and physical stresses.
In the context of this PhD thesis, knowledge on heavy metal resistance systems in thermophilic microorganisms represents the starting point for the development of biotechnological applications in the environmental field. Starting from knowledge on *T. thermophilus* HB27 which has a promiscuous resistance system for cadmium and arsenic, this review focuses on the molecular mechanisms responsible for survival and adaptation of thermophiles to toxic metals, with particular emphasis on arsenate As(V), arsenite As(III) and cadmium Cd(II). Moreover, it reports an overview on the current biotechnological applications of thermophiles for heavy metals detection, extraction and removal.

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149

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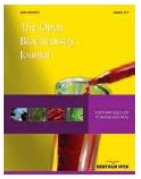

REVIEW ARTICLE

Metal-Tolerant Thermophiles: From the Analysis of Resistance Mechanisms to their Biotechnological Exploitation

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Abstract:

Extreme terrestrial and marine hot environments are excellent niches for specialized microorganisms belonging to the domains of Bacteria and Archaea; these microorganisms are considered extreme from an anthropocentric point of view because they are able to populate harsh habitats tolerating a variety of conditions, such as extreme temperature and/or pH, high metal concentration and/or salt; moreover, like all the microorganisms, they are also able to respond to sudden changes in the environmental conditions. Therefore, it is not surprising that they possess an extraordinary variety of dynamic and versatile mechanisms for facing different chemical and physical stresses. Such features have attracted scientists also considering an applicative point of view. In this review we will focus on the molecular mechanisms responsible for survival and adaptation of thermophiles to toxic metals, with particular emphasis on As(V), As(III), Cd(II), and on current biotechnologies for their detection, extraction and removal.

Keywords: Toxic metals, Resistance systems, Metal bioremediation, Thermostable biosensors, Environmental conditions, Physical stresses.

1. HEAVY METALS: TOXICITY AND TRANSFORMATION

Heavy metals are among the most persistent and toxic pollutants in the environment [1]. Even in small concentrations, they can threat human health as well as the environment because they are non-biodegradable. There is no widely agreed criterion for definition of a heavy metal. Depending on the context, this term can acquire different meanings: for example, in metallurgy a heavy metal may be defined by its density, in physics by its atomic number, and in chemistry by its chemical behavior [2]. The *International Union of Pure and Applied Chemistry* (IUPAC) does not consider these definitions correct; in this review, according to IUPAC, every "heavy" metal has the following characteristics: density exceeding 5.0 g/cm^3; general behavior as cations; low solubility of their hydrates; aptitude to form complexes and affinity towards the sulfides.

In 2010, the World Health Organization (WHO) estimated that more than 25% of total diseases were linked to environmental factors including exposure to toxic chemicals [3]. For example, lead, [Pb(II)], one of the most common heavy metals, is thought to be responsible for 3% of cerebrovascular disease worldwide [4]; while cadmium, [Cd(II)], has been classified as carcinogen by the *International Agency for Research on Cancer* (IARC) on the basis of several evidence in both humans and experimental animals [5, 6]. Furthermore, hazards associated with exposure to other metal ions like chromium [Cr(II)], mercury [Hg(I)], and arsenic [As(III) and As(V)], have been well established in the literature [7 - 12]. The risk related to heavy metal exposure depends on the concentration and time [13].

Table **1** reports the concentration limits of the most common heavy metals in drinkable water, suggested by the *World Health Organization* (WHO) and the *Environmental Protection Agency* (EPA). It also shows the possible sources of these contaminants in drinking water and the potential health effects from long-term exposure [12, 14].

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Table 1. Limit concentrations of common heavy metals in drinkable water, suggested by the *World Health Organization* (WHO) and the *Environmental Protection Agency* (EPA).

Contaminant	WHO (mg/L)	EPA (mg/L)	Potential Health Effects from Long-Term Exposure	Sources of Contaminant in Drinking Water
As	0.010	0.010	Skin damage or problems with circulatory systems, and may have increased risk of getting cancer	Erosion of natural deposits; runoff from orchards, runoff from glass and electronics production wastes
Ba	7	2	Increase in blood pressure	Discharge of drilling wastes; discharge from metal refineries; erosion of natural deposits
Cd	0.003	0.005	Kidney damage	Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints
Cr (total)	0.050	0.100	Allergic dermatitis	Discharge from steel and pulp mills; erosion of natural deposits
Cu	2	1.300	Liver or kidney damage	Corrosion of household plumbing systems; erosion of natural deposits
Hg (inorganic)	0.006	0.002	Kidney damage	Erosion of natural deposits; discharge from refineries and factories; runoff from landfills and croplands
Pb	0.010	0.015	Kidney problems; high blood pressure	Corrosion of household plumbing systems
Sb	0.020	0.006	Increase in blood cholesterol; decrease in blood sugar	Discharge from petroleum refineries; fire retardants; ceramics; electronics; solder

On the other hand, heavy metals naturally occur in the Earth's crust. They are present in soils, rocks, sediments, air and waters and can be used and modified by local microbial communities, which are actively involved in metal geochemical cycles, affecting their speciation and mobility. Many metals are essential for life because they are actively involved in almost all aspects of metabolism: as examples, iron and copper are involved in the electron transport, manganese and zinc influence enzymatic regulations. However, their excess can disrupt natural biochemical processes and cause toxicity. For these reasons, all the microorganisms have evolved resistance systems to get rid of the cell of toxic metals as well as molecular mechanisms to maintain metal homeostasis. These systems frequently rely on a balance between uptake and efflux processes [15]. Because of microbial adaptation, microorganisms can also contribute to increase toxicity levels [16, 17]. For example, several studies in Bangladesh have demonstrated that microbial processes enhance the arsenic contamination in near- and sub-surface aquifers, because arsenate-respiring bacteria can liberate As(III) from sediments, adsorptive sites of aluminum oxides or ferrihydrite, or from minerals, such as scorodite [18].

Metal biotransformation impacts human health through the food chain: examples include the oxidation of Hg(0) to Hg(II), and the subsequent methylation to methylmercury compounds, which can be accumulated by fish and marine mammals in the aquatic environment [19].

Despite their relevant toxicity, in a report of the European Commission (named "Critical-Metals in the Path towards the Decarbonisation of the EU Energy Sector"), several heavy metals such as cadmium, chromium and lead are included into the classification of critical raw materials. According to the sustainable low-carbon economic policy of EU, these metals are expected to become a bottleneck in a near future to the supply- chain of various low- carbon energy technologies [20]. Therefore, it is very important to detect and recover these heavy metals to achieve both environmental safeguard and sustainable economic strategies. Common sources of heavy metals in this context include mining and industrial wastes, vehicle emissions, lead-acid batteries, fertilizers, paints, treated timber, aging water supply infrastructures, and microplastics floating in the world's oceans [21, 22].

2. METAL RESISTANCE MECHANISMS

Microorganisms able to tolerate high levels of heavy metal ions have evolved in ore deposits, hydrothermal vents, geothermal sites, as well as in different polluted sites [23]. Metal tolerance of thermophilic Bacteria/Archaea is due to several mechanisms, many also found in mesophilic counterparts, such as: extracellular barrier, metal ion transport into and outside the cell, the utilization of toxic metal ions in metabolism or the presence of metal resistance genes with different genomic localization (chromosome, plasmid or transposon) [24].

To date, at least four main mechanisms of heavy metal resistance, schematized in Fig. (**1**), are described which can be even found in the same microorganism [24 - 31]:

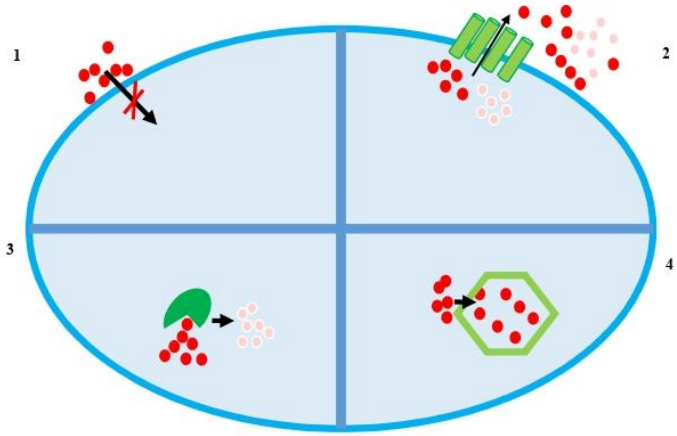

Fig. (1). Generalized illustration of the genetic mechanism of resistance to toxic metals by microorganisms: **1)** Extracellular barrier, a selectively permeable system; **2)** Efflux of metal ions; **3)** Enzymatic reduction of metal ions. **4)** Intracellular sequestration by small molecule complexing agents or metal-chelating proteins.

- Extracellular barrier;
- Active transport of metal ions (efflux);
- Enzymatic reduction of metal ions;
- Intracellular sequestration.

The cell wall or plasma membrane can prevent metal ions from entering the cell. Bacteria belonging to different taxonomical groups can adsorb metal ions by ionisable groups of the cell wall (carboxyl, amino, phosphate and hydroxyl groups) [32]. However, many metal ions enter the cell *via* the systems responsible for the uptake of essential elements: for example, Cr(II) is transported inside the cell *via* sulphate transport system [33], whereas Cd(II), Zn(II), Co(II), Ni(II) and Mn(II), enter the cells using systems of magnesium transport [34]. Moreover, As(V) is taken into cells by phosphate transport systems and As(III) has been shown to be taken up by glucose permeases [35].

Both in Gram-positive and Gram-negative bacteria the arsenic resistance system is composed by operons of 3-5 genes carried on plasmids or chromosomes; the two most common contain either five genes (*arsRDABC*), as in the plasmid R773 of the *Escherichia coli*, or three genes (*arsRBC*), as in the plasmid pI258 of *Staphylococcus aureus* [36, 37]. The *arsR* gene encodes a trans-acting repressor of the ArsR/SmtB family involved in transcriptional regulation [30], *arsB* encodes an As(III) efflux transporter [38], and *arsC* encodes a cytoplasmic arsenate reductase that converts As(V) to As(III), which is extruded outside the cell [39]. Where present, ArsD is a metallochaperone that transfers trivalent metalloids to ArsA, the arsenite-stimulated efflux ATPase [31].

2.1. Transport of Metal Ions: Efflux Systems

The majority of thermophilic microorganisms (both belonging to Archaea and Bacteria domains) are resistant to heavy metals thanks to an active transport and/or efflux of metal ions outside the cells. The genetic determinants of efflux systems can be localized on chromosomes [40] and on plasmids [41]. In most cases, the expression of metal efflux genes is transcriptionally controlled by co-transcribed metal sensor proteins [42].

In microorganisms, efflux systems consist of proteins belonging to three families: CDF (cation diffusion facilitator), P-type ATPases and RND (resistance, nodulation, cell division) [43]. CDF proteins and P-type ATPases of Gram-negative bacteria transport specific substrates through the plasma membrane into the periplasm. CDF proteins are metal transporters occurring in all the three domains of life whose primary substrates are mainly ions of divalent metals like Zn(II), Co(II), Ni(II), Cd(II), and Fe(II) and export metals through a chemiosmotic gradient formed by H^+ or K^+ [44, 45]. Most of these proteins have six transmembrane helices containing a zinc-binding site within the transmembrane domains, and a binuclear zinc-sensing and binding site in the cytoplasmic C-terminal region [46]. These proteins exhibit an unusual degree of sequence divergence and size variation (300-750 residues).

Differently from CDF-proteins, P-type ATPases transfer both monovalent or bivalent metal ions with high affinity for sulfhydryl groups (Cu(I)/Ag(I), Zn(II)/Cd(II)/Pb(II)) and use ATP hydrolysis to transport ions across cellular membranes [43]. They are composed of three conserved domains: i) a transmembrane helix bundle, allowing substrate translocation; 2) a soluble ATP binding domain containing a transiently phosphorylated aspartate residue; 3) a soluble actuator domain (AD). Those belonging to P_{1B}-type are capable to drive the efflux out of cells of both essential transition metal ions (*e.g.*, Zn(II), Cu(I), and Co(II)) and toxic metals (*e.g.*, Ag(I), Cd(II), Pb(II)) contributing to their homeostasis maintenance. In a recent study on a huge number of P_{1B}-type ATPase they have been classified into seven distinct subfamilies (1_{B-1} 1_{B-7}) but the molecular basis of metal ion specificity remains unclear [47]. Several thermophilic P_{1B}-type ATPases involved in metal efflux have been characterized. The thermophilic bacterium *Thermus thermophilus* HB27 contains in its genome three genes coding for putative P_{1B}-type ATPases: *TTC1358*, *TTC1371*, and *TTC0354*; these genes are annotated, respectively, as two putative copper transporter (CopA and CopB) and a zinc-cadmium transporter (Zn(II)/Cd(II)-ATPase) [48], involved in heavy metal resistance. *Archaeoglobus fulgidus* possesses a CopA protein driving the outward movement of Cu(I) or Ag(I) characterised by a conserved CPC metal binding site and a cytoplasmic metal binding sequence (also containing cysteine residues) at its N- and C- terminus [49].

Members of the RND family are efflux pumps, especially identified in Gram-negative bacteria that can be divided in subfamilies depending on the substrate transported; they actively export heavy metals, hydrophobic compounds, nodulation factors [50]. The heavy metal efflux (HME) RND sub-family functions for metal ion efflux powered by a proton-substrate antiport. The prototype family member from *E. coli* is CusA; it works in conjunction with the membrane fusion protein CusB and the outer-membrane channel CusC forming a tripartite complex spanning the entire cell envelope to export Cu(I) and Ag(I) [51]. The crystal structures of CusA in the absence and presence of bound Cu(I) or Ag(I) has been recently solved providing structural information [52].

To the best of our knowledge, thermophilic microbial genomes do not contain genes encoding proteins of the HME-RND family. A Blast analysis of CusA against thermophilic genomes revealed homology with integral membrane proteins of the ACR (Activity Regulated Cytoskeleton Associated Protein) family involved in drug and/or heavy metal resistance [53].

2.2. Enzymatic Reduction of Metal Ions: Metal Reductases

Many thermophilic microorganisms employ intracellular enzymatic conversions combined with efflux systems to obtain heavy metal resistance. Enzymatic reduction of metal ions can result in the formation of less toxic forms like Hg(II) reduced to Hg(0), Cr(V) converted into Cr(III) [23] or, as in the case of As(V), reduction in the more toxic As(III) which is the only form extruded by the cell.

Several thermophilic metal reductases have been described so far; for example, *Tt*ArsC from *Thermus thermophilus* HB27 is an arsenate reductase which enzymatically converts As(V) in As(III) [54] using electrons provided from the thioredoxin-thioredoxin reductase system and employing a catalytic mechanism in which the thiol group of a N-terminal cystein performs a nucleophilic attack on the arsenate [54]. As told before the arsenite is then extruded by a dedicated efflux protein.

MerA from *Sulfolobus solfataricus* is flavoprotein that catalyzes the reduction of Hg(II) to volatile Hg(0), converting toxic mercury ions into relatively inert elemental mercury [55].

The thermophilic bacteria isolated from various ecological niches can also reduce a broad spectrum of other heavy metal ions such as Cr(V), Mo(VI) and V(V) [56] that serve as terminal acceptors of electrons during their anaerobic respiration [57].

These systems are generally finely regulated by specific transcription factors. As an example, the transcription of the arsenic resistance system of *T. thermophilus* HB27 is regulated by *Tt*SmtB, a protein belonging to the ArsR/SmtB family which acts as the As(V) and As(III) intracellular sensor [30]. In the absence of metal ions, the protein binds to regulatory regions upstream of *TTC1502,* encoding *Tt*ArsC, and *TTC0354*, encoding the efflux membrane protein *Tt*ArsX, (a P_{1B}- type ATPase, see above). In a recent study from our group it was demonstrated that *Tt*ArsX and *Tt*SmtB are also responsible for Cd(II) tolerance [58].

2.3. Metal Intracellular Sequestration

Another common mechanism to inactivate toxic metal ions is the intracellular sequestration or the complexation of metal ions by various compounds in the cytoplasm. The metallothioneins and phytochelatins are two classes of peptides

rich in cysteine residues which bind metal ions through the sulfhydrylic groups [59].

Metallothioneins constitute a superfamily of ubiquitous cytosolic small (25–82 amino acids), cysteine-rich (7–21 conserved Cys residues) proteins able to bind metal ions, mainly Cd(II), Zn(II) and Cu(I), *via* metal-thiolate clusters in the absence of aromatic amino acids and histidine residues [60]. They are multifunctional proteins whose synthesis is stimulated by heavy metals and other environmental stressors; based on this latter evidence the metallothionein promoter of *Tetrahymena thermophila* has been employed in the development of a *whole-cell* biosensor for the detection of heavy metals [61].

Among prokaryotes, the ability to synthesize metallothionein has been demonstrated in the cyanobacterium *Synechococcus* sp. PCC 7942 which contains two genes *smtA* and *smtB* inducible by Cd(II) and Zn(II). The peptide contained fewer cysteine residues than the eukaryotic metallothionein [62].

3. APPLICATIONS IN BIOTECHNOLOGY

A detailed understanding of the molecular mechanisms responsible for resistance to toxic metals in metal-tolerant microorganisms is also crucial for a potential use in the environmental monitoring of metal contamination and to set up bioremediation processes, the most promising being biosorption and removal as insoluble complexes Fig. (**2**).

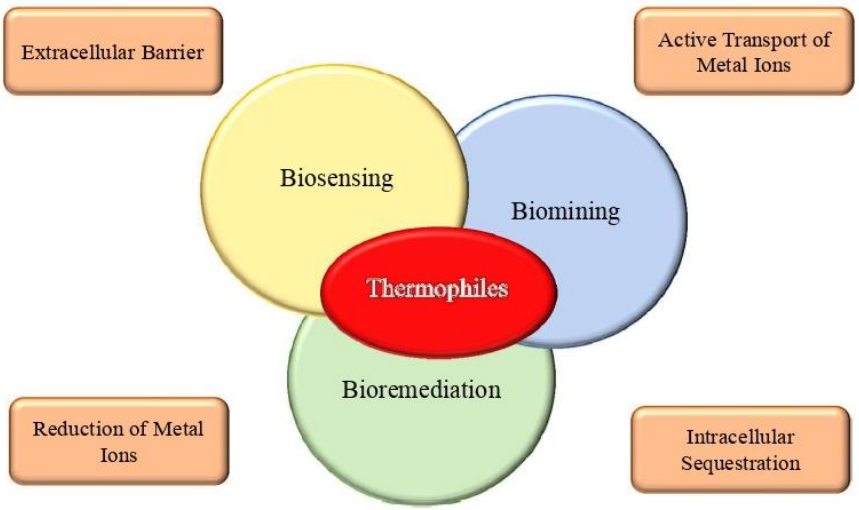

Fig. (2). Schematic representation of potential applications in biotechnology of metal-tolerant thermophiles.

The traditional approach for monitoring the environmental pollution is based on chemical or physical analysis and allows highly accurate and sensitive determination of the exact composition of any sample. These analyses require specialized and expensive instrumentations, as the ICP-MS (*Inductively Coupled Plasma - Mass Spectrometry*) which, to date, is the most adopted technique for detecting heavy metals [63].

The need for accurate, not expensive, on-site and real-time measurements has led to the development of sensors based on biomolecules and nanomaterials [63 - 66]. Biosensors are analytical devices which integrate a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of the analyte [67 - 69]. The use of biological molecules is a considerable advantage in the sensor field, because of their high specificity: these sensors are based on the specific interaction between enzymes and their substrates, antibodies and antigens, target molecules to their receptors, or the high affinity of nucleic acid strands to their complementary sequences [67]. Nevertheless, some biomolecules can be too labile for the exploitation on the marketplace. In this context, the biomolecules of the thermophiles are more stable at high temperatures than the mesophilic counterparts. In fact they have already been exploited in biotechnology, as demonstrated by the development of the polymerase chain reaction (PCR) and the use of thermozymes in many industrial applications [70 - 73].

The recent developments in nanotechnology have also opened new horizons for biosensing: nanomaterials are attractive because of their unique electrical, chemical and physical properties (*i.e.* size, composition, conductivity, magnetism, mechanical strength, and light-absorbing and emitting). The most studied of them, carbon nanotubes (CNTs), graphene, metal nanoparticles (MNPs), and quantum dots (QDs), have been especially targeted for developing novel biosensors [64, 73 - 76]. An example of a successful match between thermophilic biomolecules and nanomaterial for the development of a heavy metal biosensor is reported by Politi *et al*. [77, 78]. In this work, *Tt*ArsC, the arsenate reductase from *T. thermophilus* HB27 [54] was conjugated to polyethylene glycol-stabilized gold nanospheres. The new nanobiosensor revealed high sensitivities and limits of detection equal to 10 ± 3 M^{-12} and 7.7 ± 0.3 M^{-12} for As(III) and As(V), respectively [77, 78].

A detailed understanding of the molecular mechanisms responsible for resistance to toxic metals is crucial to develop whole cell biosensors for the detection of chemicals in the environment so that not only thermophilic biomolecules, but also thermophilic microorganisms can constitute heavy metal biosensors. Many whole-cell biosensors for metal ions detection have been already described in the literature based on the realization of reporter systems containing regulatory cis-acting sequences interacting with the cognate transcriptional metal sensor repressor; however, to date, there are few reports on thermophilic whole cell biosensors [79, 80].

In the work by Poli *et al*. [57], *Anoxybacillus amylolyticus*, an acidothermophilic bacterium isolated from geothermal soil samples in Antarctica, was observed to be resistant to metals like Ni(II), Zn(II), Co(II), Hg(II), Mn(II), Cr(VI), Cu(II) and Fe(III). A decrease in α-amylase activity, correlated with a decrease in α-amylase production, was observed in the presence of heavy metals: it is speculated to use it as a toxicological indicator of heavy metals in a potential microbial bioassay employing whole cells [57].

Biomining and bioremediation represent a new branch of biotechnology, named biometallurgy, addressed at heavy metal recovery including the processes that involve interactions between microorganisms and metals or metal-bearing minerals [81]. Biomining refers to the exploitation of microorganisms to extract and recover metals from ores and waste concentrates (the term is often used synonymously with *bioleaching* when the metals are solubilized during the process); on the other hand, bioremediation focuses on the transformation of a toxic substance into a harmless or less toxic one from contaminated sites [82, 83]. In this contest, among the heavy metals resistance systems developed by microorganisms, and in particular by thermophiles, biosorption and bioaccumulation are emerging as promising low-cost methodologies for bioremediation [84, 85]. Biosorption and bioaccumulation are two processes that consist into the ability of microorganisms to accumulate heavy metals from wastewater through metabolic pathways or physical-chemical uptake; but while biosorption is a passive process depending on the composition of the cellular surface and following a kinetic equilibrium, bioaccumulation is an energy driven process and requires an active metabolism [59].

Several examples of biosorption and bioaccumulation are provided by microorganisms belonging to *Geobacillus sp.*, which are highly tolerant to Cd(II), Cu(II) and Zn(II) [86 - 88]. *Özdemir, S. et al,* indicated a *G. toebii subsp. decanicus* as an efficient viable biosorbent for heavy metals. This study clearly shows that thermophiles can be used for removal and recovery of heavy metals from industrial wastewater [89].

The interest into the potential applications of heavy metal resistant thermophiles has led to the development of tools and assays for screening them on lab scale [61, 90 - 92], with the final goal to design and set up bacterial bioprocesses on the industrial scale [93 - 95]. Table **2** summarizes some examples of bioprocesses employing heavy metals performed by thermophiles.

Table 2. Examples of heavy metals bioprocesses performed by thermophiles.

Heavy Metals	Microrganisms	Bio Processes	References
$CuFeS_2$	*Leptospirillum ferriphilum* *Acidithiobacillus caldus*	Bioleaching	*Zhou H.B. et al, 2009* [94]
Fe(II)	*Sulfobacillus sp.*	Bioleaching	*Hawkes R. et al, 2006* [92]
As(III), As(V)	*Thermus thermophilus*	Biosensing	*Politi J. et al, 2015* [77]
Ni(II), Zn(II), Co(II), Hg(II), Mn(II), Cr(VI), Cu(II) and Fe(III)	*Anoxybacillus amylolyticus*	Biosensing	*Poli et al, 2008* [57]
Cd(II)	*Tetrahymena thermophila*	Biosensing	*Amaro F. et al, 2011* [61]
Cd(II), Cu(II), Ni(II), Mn(II), Zn(II)	*Geobacillus toebii subsp. decanicus* *Geobacillus thermoleovorans subsp. stromboliensis*	Biosorption	*Özdemir, S. et al, 2012* [86]

(Table 2) contd.....

Heavy Metals	Microrganisms	Bio Processes	References
Cd(II)	*Geobacillus stearothermophilus* *Geobacillus thermocatenulatus*	Biosorption	*Hetzer, A. et al, 2006* [87]
Fe(III), Cr(III), Cd(II), Pb(II), Cu(II), Co(II), Zn(II), Ag(I)	*Geobacillus thermodenitrificans*	Biosorption	*Chattereji S.K. et al, 2010* [88]

4. RESULT

Metal-tolerant thermophiles exhibit metabolic and physiological features that distinguish them from other major life groups due to their adaptation to extreme environments.

CONCLUSION

Most of the knowledge regarding mechanisms of adaptation/resistance to toxic metals has been discovered using traditional microbiological/biochemical techniques and thanks to the use of genomics, transcriptomics, proteomics as well as to the recent development of genetic tools for many of these organisms. With the advent of next-generation sequencing technologies, comparative genomics and metagenomics projects, it appears that even novel metabolic features can be discovered, further expanding our understanding of environmental microbiology. Such an integrated view opens to new opportunities for biotechnological applications in commercially relevant processes such as the monitoring of metal concentrations in the environment, the recovery of precious and strategic metals and the setup of microbial-based remediation strategies.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Chapter [3]: Development of *Tt*ArsC based biosensors

The arsenate reductase from *T. thermophilus* (*Tt*ArsC) is able to reduce As(V) into As(III) using reducing equivalents passing through the Thioredoxin reductase-Thioredoxin (TR–Trx) system. The catalytic mechanism involves the thiol groups of three Cys residues (Cys7, Cys83, Cys90): Cys7 performs a nucleophilic attack on the As(V), while the formation of a disulphide bond between Cys83 and Cys90 provides electrons for As(V) reduction, followed by As(III) release. The recycling of the enzyme is guaranteed by the intracellular TR-Trx reducing system.

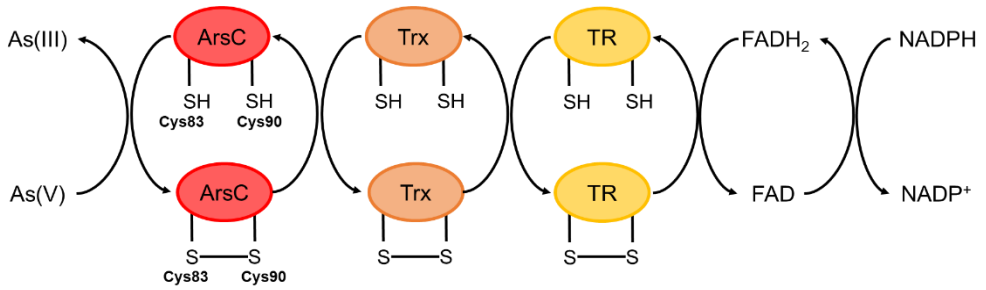

Figure [3.1] Catalytic mechanism of *Tt*ArsC coupled with the TR-Trx system. It is highlighted the redox cascade involving the TR–Trx system and the *Tt*ArsC residues Cys83 and Cys90, forming a disulphide bond. Cys7 residue which performs the nucleophilic attack on the As(V) is not shown.

*Tt*ArsC also displays a phosphatase activity, thanks to the presence of an anion binding site known as P-loop (or the CX5R motif) and an Asp-Pro sequence (Asp acts as catalytical acid–base), both typically conserved in low molecular weight protein tyrosine phosphatases (LMW PTPases).

This enzyme is very resistant to chemical-physical denaturation, such as high temperatures and elevated concentrations of guanidine. Nonetheless, it is the most thermostable arsenate reductase characterized to date: it is fully active after 90 min at 80 °C, and still maintains 50% activity after 30 min at 87 °C. These characteristics make it a good candidate for several application, including the development of biosensors. In particular, the literature is full of proof of concept examples for the development of arsenic biosensors, which often remain on a lab scale. Major bottlenecks regard the stability of the biomaterials, the techniques required for immobilization on supports, the detection methods of analytes (optical, electrochemical, gravimetric, etc.), the sensitivity and the specificity of the system; not least, also the costs for an industrial scale production.

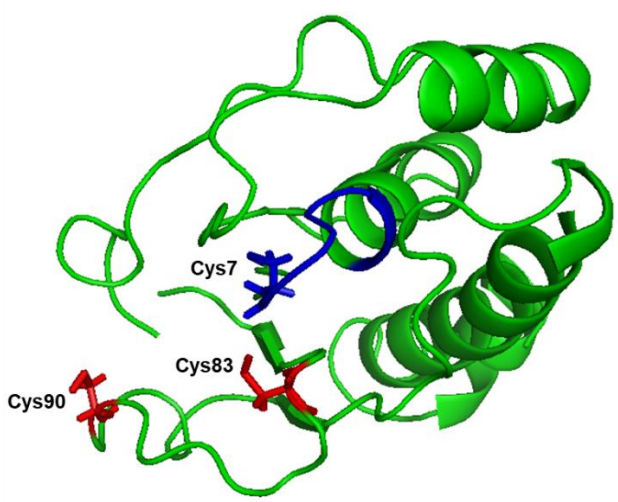

Figure [3.2] 3D model of *Tt*ArsC. The P-loop including the Cys7 residue is reported in blue; the other two Cys residues (Cys83 and Cys90) are coloured in red.

This chapter regards the exploitation of *Tt*ArsC as the biorecognition component of an arsenic biosensor:

- In Paper I we describe the setup of an innovative biosystem realized through the genetic fusion of *Tt*ArsC to a protein with adhesive properties (the fungal hydrophobin Vmh2). The genetic fusion with Vmh2 allows a one-step immobilization of the chimeric protein on hydrophobic supports, just by deposition. Therefore, one aim was to overcome the problem related to the weakness of the biomaterial and immobilization technique. In particular, two chimeric proteins have been produced ArsC-Vmh2 and Vmh2-ArsC, that have been biochemically characterized and immobilized on either polystyrene and gold electrodes to detect As(III) via electrochemical procedures. The main methodologies applied in this paper are: heterologous expression and purification of recombinant chimeras from *Escherichia coli;* renaturation from inclusion bodies; Western Blot; reductase activity assay (using As(V) as substrate) and phosphatase activity assay (using p-nitrophenyl phosphate (pNPP) as substrate); immobilization on polystyrene and gold supports; Quartz Cristal Microbalance (QCM) analyses and electrochemical analyses, i.e, Cyclic Voltammetry (CV) and Square Wave Voltammetry (SWV).
- In Paper II we report the realization of an As(V) detection system based on the inhibition of *Tt*ArsC phosphatase activity by As(V).

Object of this study is the evaluation of inhibition of phosphatase activity by arsenic. We found that arsenic behaves as non-competitive inhibitor. Therefore, the phosphatase activity of *Tt*ArsC has been deeply investigated in relation to its inhibition by arsenic, especially by As(V). In particular, we also evaluated if the inhibitory response to As(V) was preserved in the presence of other compounds (heavy metals, salts, pesticides), with the aim to assess the specificity of the system. The specific inhibition by As(V) sets the condition for the development of an easy to use optical As(V) inhibition-based biosensor. The main methodologies applied in this paper are based on the phosphatase activity assay, using the pNPP as substrate; in particular we developed: Michaelis-Menten kinetics and Lineweaver–Burk double-reciprocal plots, an As(V) dose-response curve, graphs of retained activity in presence and co-presence of other compounds.

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Self-assembling thermostable chimeras as new platform for arsenic biosensing

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The correct immobilization and orientation of enzymes on nanosurfaces is a crucial step either for the realization of biosensors, as well as to guarantee the efficacy of the developed biomaterials. In this work we produced two versions of a chimeric protein, namely ArsC-Vmh2 and Vmh2-ArsC, which combined the self-assembling properties of Vmh2, a hydrophobin from *Pleurotus ostreatus*, with that of *Tt*ArsC, a thermophilic arsenate reductase from *Thermus thermophilus*; both chimeras were heterologously expressed in *Escherichia coli* and purified from inclusion bodies. They were characterized for their enzymatic capability to reduce As(V) into As(III), as well as for their immobilization properties on polystyrene and gold in comparison to the native *Tt*ArsC. The chimeric proteins immobilized on polystyrene can be reused up to three times and stored for 15 days with 50% of activity loss. Immobilization on gold electrodes showed that both chimeras follow a classic Langmuir isotherm model towards As(III) recognition, with an association constant ($K_{As(III)}$) between As(III) and the immobilized enzyme, equal to 650 (± 100) L mol^{-1} for ArsC-Vmh2 and to 1200 (± 300) L mol^{-1} for Vmh2-ArsC. The results demonstrate that gold-immobilized ArsC-Vmh2 and Vmh2-ArsC can be exploited as electrochemical biosensors to detect As(III).

Arsenic (As) is a toxic metalloid widespread in soil, water and air, harmful to humans and the environment[1,2]; due to its toxicity, one biotechnological challenge is centred on the development of biosensors to monitor its concentration in the environment[3]. Among species found in natural waters, the inorganic forms of As(III) and As(V) are those predominant with enhanced toxicity and greater mobility[4]. Microbial activities play important roles in the mobilization of arsenic[5–7], and comprehensive knowledge on the molecular basis of arsenic metabolism/tolerance is a key step for developing efficient and selective arsenic biosensors.

The arsenic resistant thermophilic bacterium *Thermus thermophilus* HB27 owns an arsenate reductase (*Tt*ArsC) capable of reducing As(V) to As(III), which is then extruded outside the cell by a membrane P_{1B} type ATPase[8]; both proteins are finely regulated at transcriptional level by the metal responsive transcriptional repressor *Tt*SmtB[9,10]. *Tt*ArsC belongs to a subfamily of thioredoxin-coupled arsenate reductases, whose paramount protein is ArsC from *Staphylococcus aureus* plasmid pI258, characterised by the presence of three redox active cysteines, one performing the nucleophilic attack to the substrate and the other two that form a Cys–Cys disulphide bond upon reduction of As(V) into As(III)[11]. Members of this subfamily are also endowed with phosphatase activity because they contain a P-loop (CTHNSAR) homologous to that of low molecular weight protein tyrosine phosphatases (LMW PTPase) whose consensus sequence is CXGNXCR. Messens and co-workers proposed that these ArsCs evolved from LMW PTPase by a change of mechanism, that maintained the oxyanion substrate binding[12]. Consequently, to perform reductase activity they require the action of disulphide cascades and interaction with thioredoxin reductase (Tr), thioredoxin (Trx) and NADPH as redox partners, while to perform phosphatase activity they use mainly amino acids of the phosphate binding loop[11]. During the reduction reaction, a major conformational change occurs in ArsC upon oxidation, which is required to transport the oxidative equivalents from the As(V) in the P-loop to the surface of the enzyme[12].

In *Tt*ArsC, the oxyanion binding site is conserved and includes the catalytic nucleophile Cys7, which is involved in both arsenate and phosphate binding. Nevertheless, its phosphatase activity is much weaker than that reported for ArsC from *S. aureus* pI258, probably because of differences in the amino acid composition of the active-site loop[13]. The other two conserved Cys residues (Cys82 and Cys89), essential for the reduction of

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the arsenate substrate, are spatially separated from the P-loop. To date, *Tt*ArsC is one of the most thermostable arsenate reductases characterised[13].

Thermophilic enzymes/proteins are known to be more resistant to harsh conditions than their mesophilic counterparts, in fact they are often employed in many biotechnological applications[14–16], such as degradation of polysaccharides of industrial interest[17,18], antioxidants[19], molecular biology tools[20,21]; and biosensing[8,22,23]. *Tt*ArsC, thanks to its thermophilic nature, is highly resistant to changes in pH, temperature and ionic strength, and was employed as component of an optical biosensor able to detect both As(V) and As(III) at low concentrations[24,25].

In recent years it has emerged that a key step for increasing the performances of biosensors is represented by the biological interfacing of materials[26,27]. In particular, electrochemical biosensors are based on the efficient immobilization of enzymes on electrode surfaces. This immobilization technique must rely on effective functionalization strategies in order to preserve the recognition and/or catalytic ability of enzymes towards the targeted substrate[28–30]. In the case of enzyme-based arsenic biosensors, molybdenum-containing arsenite oxidases have been most exclusively investigated owing to its ability to electroenzymatically oxidize arsenite on electrodes[3,31–33]. Other examples of arsenic biosensors have been based on the inhibition properties of arsenic towards certain type of enzyme electrocatalytic activity[34].

In bio-devices the specific properties and performances of bio/non-bio interfaces are also crucial features, therefore the development of biosensors based on suitably engineered self-assembling amyloid fibrils constitutes a promising opportunity to fulfil this task[35]. Self-assembling proteins are considered as a fundamental and green strategy to build hierarchical structures in hybrid functional assemblies[36]; in this context, hydrophobins (HFBs) are small fungal proteins (≈ 20 kDa) able to assemble spontaneously into amphiphilic monolayers at hydrophobic/hydrophilic interfaces. These amphiphilic proteins can be grouped into two classes based on the spacing of eight conserved cysteine residues and the nature of the amphipathic monolayers that they form. Class I HFBs are amongst the first proteins recognized as functional amyloids[37]; they form fibrillar structures which are extremely robust and can be disassembled only in strong acids. Vmh2 from *Pleurotus ostreatus*, is a class I HBF, known to self-assemble into stable films, able to change the wettability of surfaces and to strongly adsorb other proteins in their active form[38–41]. Thanks to its characteristics, Vmh2 has already been employed in the construction of chimeric proteins via genetic fusions with different proteins: the enzyme glutathione-*S* transferase (GST), which was used to quantify toxic compounds in aqueous environmental samples[42]; the green fluorescent protein (GFP) for the development of a biosensor to monitor thrombin in plasma samples[43]; the laccase POXA1b from *P. ostreatus* for the detection of phenolic compounds in different matrices[44]; and the antimicrobial peptide LL-37 for the development of anti-bacterial surfaces[45].

In this context, this work aimed at realizing arsenic biosensing by developing a chimeric protein which combines the recognition properties and the straightforward stability of *Tt*ArsC with the self-assembling properties of the hydrophobin Vmh2.

Results and discussion

Design of chimeric proteins. In order to develop a biosensor with increased biosensing and surface adhesive properties, two different chimeric genes were designed: one coding for *Tt*ArsC at the N-terminal and Vmh2 at the C-terminal (ArsC-Vmh2), and a second coding for Vmh2 at the N-terminal and *Tt*ArsC at the C-terminal (Vmh2-ArsC). Both chimeric genes contained a sequence encoding a flexible linker of 15 amino acids between the two proteins, and a His-tag at the C-terminal, preceded by a thrombin cleavage site. The I-TASSER tool[46,47] was used to generate the 3D models of chimeras, as well as those of the native Vmh2 and *Tt*ArsC. The confidence scores (C-score) of the predicted models for ArsC-Vmh2 and Vmh2-ArsC are − 3.72 and − 2.80, respectively, suggesting that they can be considered reliable.

As shown (Supplementary Fig. S1a), the obtained model of HFB overlaps that of the native hydrophobin Vmh2, previously proposed by Pennacchio et al.[48]. The models of ArsC-Vmh2 and Vmh2-ArsC show that the arsenate reductase and the hydrophobin are independently folded in both the chimeras; the 3D model of *Tt*ArsC substantially overlaps the corresponding moiety in both chimeras (Supplementary Fig. S1b,c), suggesting that the folding of the arsenate reductase is preserved; on the other hand, ArsC-Vmh2 and Vmh2-ArsC models present differences in the Vmh2 region, which appears as a disordered loop in the first chimera, while it is organized in α-helixes in Vmh2-ArsC (Fig. 1). Moreover, Vmh2 folding in the models of both chimeras differ from that of the native HFB. Hydrophobins are known to be plastic and highly flexible proteins that can modify their structures upon changing external conditions or interaction with other proteins[49–51]; however, 3D structural studies are necessary to draw definitive conclusions on how Vmh2 folding influences the chimeras adhesive properties.

Production of chimeric proteins. The recombinant protein production in *E. coli* was conducted inducing gene expression during the exponential growth phase. The estimated amount of protein present as inclusion bodies (mg/liter of culture) was ~ 24 mg for Vmh2-ArsC and ~ 12 mg for ArsC-Vmh2, respectively. To recover refolded proteins from the inclusion bodies, three different conditions were exploited (Table 3, "Methods" section): the best renaturation procedure resulted to be condition A, consisting in a refolding step in Tris–HCl (A_R and C_R refolding buffers), followed by dialysis in Tris–HCl (A_D dialysis buffer). Vmh2-ArsC is not only more expressed in *E. coli*, but it is also better recovered: a higher yield of refolded Vmh2-ArsC was obtained in comparison to ArsC-Vmh2 (~ 15.5 mg vs ~ 4.5 mg in condition A; ~ 4.1 mg vs ~ 1.1 mg in condition B; ~ 1.6 mg vs ~ 0.1 mg in condition C) (Supplementary Fig. S2a). The differences in expression and recovery yields can be explained by a diverse aggregation propensity of the unfolded chimeras as estimated by the TANGO tool[52–54]; in particular, ArsC-Vmh2 has a higher tendency to aggregation than Vmh2-ArsC; indeed, SDS PAGE analysis shows that the electrophoretic pattern of ArsC-Vmh2 contains bands at higher molecular weight in comparison to Vmh2-ArsC. Protein identity was also confirmed by Western blot analysis (Supplementary Fig. S2b,c and

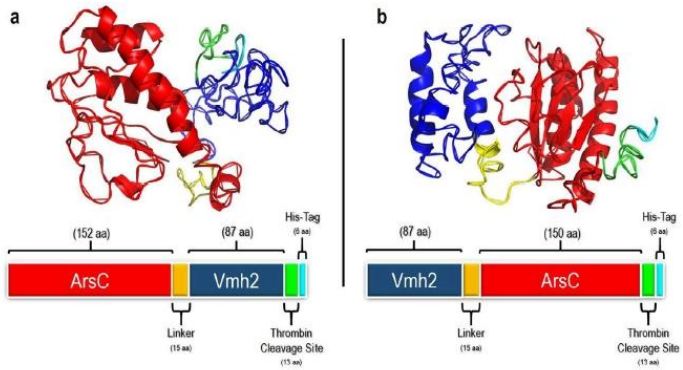

Figure 1. 3D models and schematic organization of the chimeric proteins. (**a**) ArsC-Vmh2 and (**b**) Vmh2-ArsC. In both panels ArsC is reported in red; Vmh2 in blue; the linker in yellow, the thrombin cleavage site in green and the His-tag in ciano.

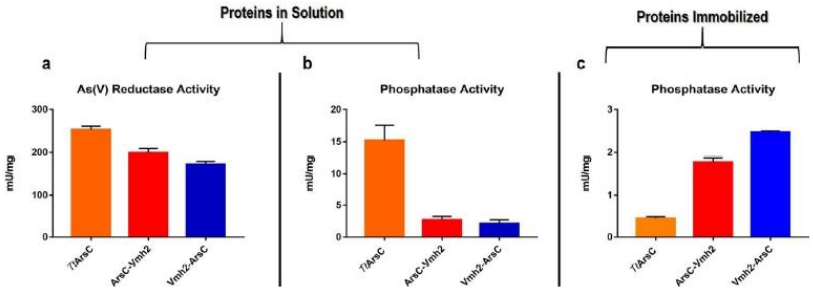

Figure 2. Graphical representation of the specific activities of *Tt*ArsC (yellow), Arsc-Vmh2 (red) and Vmh2-ArsC (blue). (**a**) As(V) reductase activity assay; (**b**) phosphatase activity assay; (**c**) phosphatase activity assay on the immobilized proteins.

Supplementary Fig. S4). Since higher amounts of both refolded chimeras were obtained using a Tris–HCl buffer (~15.5 mg vs ~4.5 mg), all the following characterizations were performed on chimeras renatured using the condition A.

Activity of the chimeric enzymes. In order to verify whether the chimeric proteins were active enzymes, arsenate reductase activity assays were performed using recombinant purified *Tt*ArsC as control[13]. Enzyme assay relies on the use of a redox cascade to recycle the enzyme. The enzymatic activity was measured by coupling the oxido/reducing system NADPH/Tr/Trx to chimeras and following the decrease of NADPH at 340 nm[12]. This assay was previously set up for *Tt*ArsC and use a hybrid system composed by the recombinant thermophilic *Ss*Tr of *S. solfataricus* and the recombinant Trx from *E. coli*, both stable at 60 °C[55,56]. Chimeric proteins exhibit a specific activity (ArsC-Vmh2 0.20 ± 0.01 U/mg; Vmh2-ArsC 0.17 ± 0.01 U/mg) comparable to that of *Tt*ArsC (0.25 ± 0.01 U/mg) (Fig. 2a; Supplementary Table S1).

The activity of the chimeras was also investigated through phosphatase activity assays using pNPP as substrate. The arsenate reductase moiety in the chimeras is in fact endowed with vestigial phosphohydrolase activity according to the hypothesis that the oxidoreductase function evolved from phosphatases in an oxidizing atmosphere[57,58]. This assay is much simpler and does not require other proteins. Notably, the phosphatase activity assay showed that both chimeras have similar specific activities (ArsC-Vmh2 2.7 ± 0.5 mU/mg; Vmh2-ArsC 2.2 ± 0.5 mU/mg) (Fig. 2b) but almost fivefold lower than that detected for *Tt*ArsC (15 ± 2 mU/mg) (Fig. 2b; Supplementary Table S1). This latter result highlights a difference in the activity of the chimeras in comparison to *Tt*ArsC, that was not observed when the activity was measured in the reductase assay. This fact can be explained hypothesizing that diverse conformational changes occur upon binding of the two substrates; in fact arsenate

	(μg) spotted			
Yield of immobilization (%)	2.5 (%)	5 (%)	10 (%)	20 (%)
ArsC-Vmh2	100	100	100	54 ± 5
Vmh2-ArsC	100	100	100	56 ± 5
*Tt*ArsC	100	76 ± 5****	60 ± 5****	N.A

Table 1. Yield of immobilization of ArsC-Vmh2 and Vmh2-ArsC compared to *Tt*ArsC on polystyrene plates. *N.A.* not analysed. Statistical analysis was performed through the ordinary one-way ANOVA on GraphPad Prism 7.00; significant differences of TtArsC in comparison to chimeras are indicated as: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. The variability is reported as standard deviation.

and the synthetic *p*-nitrophenylphosphate are structurally very different and can influence chimeras structural rearrangement in different way.

These results prove that the enzymatic properties of *Tt*ArsC are maintained in the chimeras and hence either ArsC-Vmh2 and Vmh2-ArsC are good candidates for arsenic sensing.

Immobilization on polystyrene plate. In order to investigate on the adhesive properties of the chimeric proteins, we assessed their ability to adsorb on polystyrene; for this purpose, 100 μL of chimeras were spotted on a multiwell polystyrene plate at different concentrations and the amount of immobilized protein determined. The best immobilization conditions were chosen comparing the yields calculated as described below (Methods-Immobilization on polystyrene plate). Table 1 shows that both ArsC-Vmh2 and Vmh2-ArsC, despite their structural differences in the HFB domain, are adsorbed with 100% efficiency using different amount of proteins; as expected, this value is higher than that observed for native *Tt*ArsC (yield of immobilization 60% for 10 μg of protein) proving that Vmh2 moiety contributes to enzyme immobilization on hydrophobic surfaces.

Phosphatase activity of immobilized chimeras. The activity and the stability of the immobilized chimeric proteins were evaluated by measuring phosphatase activity in comparison to the immobilized native *Tt*ArsC. The specific activities were calculated on the amount of enzyme adhered on polystyrene. Interestingly, the specific activity of immobilized chimeras (ArsC-Vmh2 1.8 ± 0.1 mU/mg; Vmh2-ArsC 2.47 ± 0.03 mU/mg) resulted to be almost 5 times higher than the immobilized *Tt*ArsC (0.44 ± 0.04 mU/mg) suggesting that Vmh2 domain is critical for the adhesion (Fig. 2c; Table S1). Furthermore, the higher specific activity of Vmh2-ArsC with respect to that of ArsC-Vmh2 suggests that the catalytic moiety in Vmh2-ArsC is better exposed upon immobilization; this observation becomes more consistent also considering that when the chimeras are not immobilized, their specific activities are comparable (see above).

The functional stability of the immobilized chimeric proteins was investigated both in terms of their aging and reuse. In particular, chimeras were adsorbed on polystyrene plates and stored for 1, 4, 7 and 15 days at 4 °C; then the residual phosphatase activity was measured. Figure 3 shows that ArsC-Vmh2 retains more activity than Vmh2-ArsC in the early days, but after 15 days both chimeras still keep about 50% of their own activity. Therefore, although Vmh2-ArsC exhibits a higher specific activity, ArsC-Vmh2 results to be more stable in the first week of storage. Furthermore, the phosphatase activity assay was repeated 4 times on the same immobilized chimeras at 1-day interval; the results indicate that both chimeras can be employed up to the third assay, with ArsC-Vmh2 keeping 47% of its specific activity and Vmh2-ArsC 54%.

Altogether these results indicate that either ArsC-Vmh2 and Vmh2-ArsC can be efficiently immobilized and maintain their catalytic function for several days.

Immobilization on gold. Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) experiments were performed in order to investigate the immobilization of ArsC-Vmh2, Vmh2-ArsC and the native *Tt*ArsC enzymes on gold-coated piezoelectric quartz crystals. A typical QCM-D profile for the three enzymes is displayed in Fig. 4a. The starting baseline corresponds to the continuous flowing of the buffer solution. As soon as Vmh2-ArsC, ArsC-Vmh2, and the native *Tt*ArsC adsorption occurs on gold surfaces, a respective frequency decrease of 82, 60 and 8 Hz is observed.

The stable interaction between ArsC-Vmh2 or Vmh2-ArsC and gold results in a stable frequency signal, even when the buffer is passed through the QCM-D cell during additional 60 min. A respective 6% and 12% frequency decrease was observed for Vmh2-ArsC and ArsC-Vmh2. This confirms the excellent stability of the Vmh2-gold interaction over time, considering that a change of the nature of the buffer in the chamber might also induce a slight decrease in frequency. On the contrary, the frequency signal corresponding to the native *Tt*ArsC exhibit 38% decrease, underlining the importance of the Vmh2 domain in stabilizing the enzyme immobilization on gold.

In parallel, the increase of the dissipation factor in the case of ArsC-Vmh2 and Vmh2-ArsC reflects a decrease in the rigidity of the gold surface, which likely arises from the homogenous formation of ArsC-Vmh2 and Vmh2-ArsC enzyme layer at the surface of gold. Figure 4b displays the plot of the mass of ArsC-Vmh2 and Vmh2-ArsC adsorbed at the surface of the gold-coated quartz as a function of time. The mass uptake of ArsC-Vmh2 and Vmh2-ArsC was estimated using the Sauerbrey Eq. (1)

$$\Delta m = -C\Delta f_n/n, \quad (1)$$

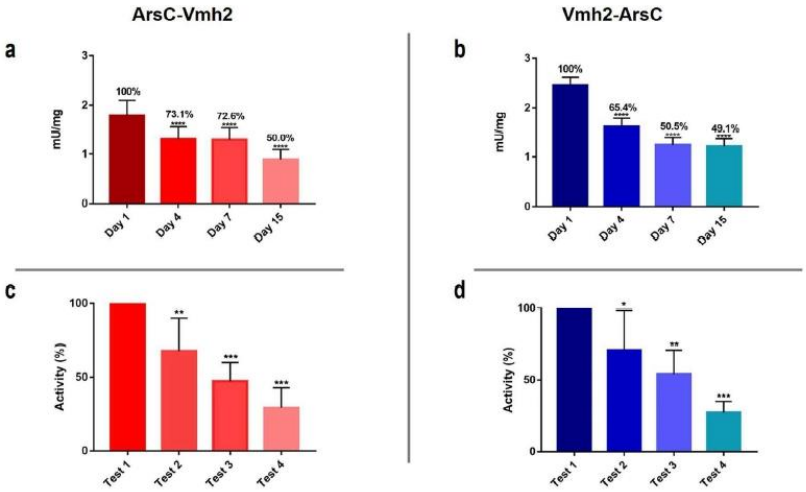

Figure 3. Stability evaluation of immobilized ArsC-Vmh2 (red) and Vmh2-ArsC (blue). (**a**,**b**) Functional stability of ArsC-Vmh2 and Vmh2-ArsC stored at 4 °C up to 15 days. (**c**,**d**) Residual activity of ArsC-Vmh2 and Vmh2-ArsC after *n* assays on the same immobilized proteins. Statistical analysis was performed through the ordinary one-way ANOVA on GraphPad Prism 7.00; significant differences are indicated as: $*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$.

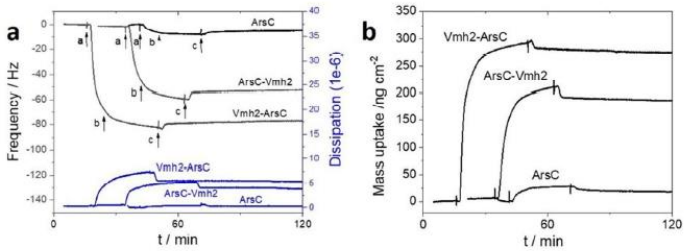

Figure 4. Immobilization on gold. (**a**) QCM-D profile (shifts in resonant frequency (black curve) and in dissipation (blue curve) vs. time) for the immobilization of ArsC-Vmh2, Vmh2-ArsC and ArsC (5th overtone data) before and (**a**) after injection of 0.04 mg mL^{-1} enzyme solution in Tris–HCl 50 mM, Gu-HCl 0.15 M, DTT 1 mM buffer pH 7.5 for 10 min, (**b**) stop of the flow for 20 min and (**c**) restart of the flow of Tris–HCl buffer; (**b**) Plot of enzyme mass uptake versus time calculated from the Sauerbray Eq. (1) for the 5th overtone.

where C is the mass sensitivity; $C = 17.7$ ng cm^{-2} Hz^{-1} at $f_1 = 5$ MHz and n is the accordant overtone number. Curves for each overtone show an average mass of immobilized ArsC-Vmh2 and Vmh2-ArsC of 187 ± 22 and 275 ± 5 ng cm^{-2} respectively. Considering a 50% degree of hydration for the enzyme layer[59], this would correspond to a ArsC-Vmh2 and Vmh2-ArsC surface density of 4.3 and 6.4 pmol cm^{-2} respectively. As expected, a low amount of native *Tt*ArsC (0.7 pmol cm^{-2}) was adsorbed on the surface of gold. In addition to high amount of immobilized enzymes, it is also noteworthy that, according to the initial mass uptake evolution, an initial rate of deposition of 2.4 and 1.0 pmol cm^{-2} min^{-1} was measured for Vmh2-ArsC and ArsC-Vmh2 respectively. This rate is almost ten-times higher compared to the simple adsorption of the native enzyme (0.25 pmol cm^{-2} min^{-1}), hypothesizing that *Tt*ArsC, Vmh2-ArsC and ArsC-Vmh2 might have the same flow rate into the cell.

Electrochemical As(III) biosensor. Vmh2-ArsC and ArsC-Vmh2-modified gold electrodes were then investigated towards arsenate and arsenite capture and electrochemical detection by anodic stripping voltam-

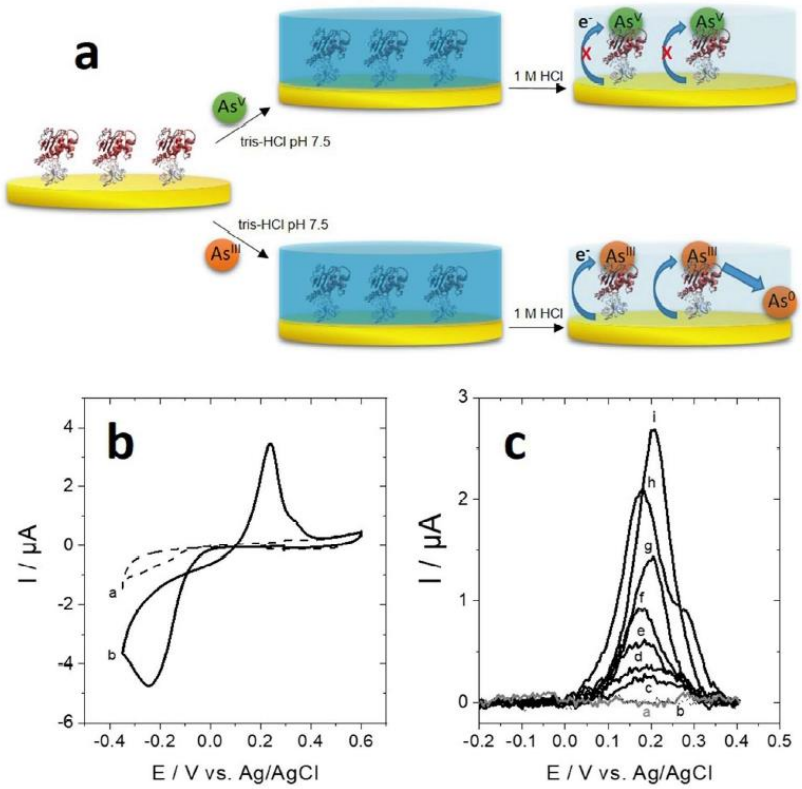

Figure 5. Electrochemical arsenic biosensor. (**a**) Schematic representation of the binding of As(III) and As(V) at Vmh2-ArsC modified gold electrodes. Down: (**b**) CV of the (**a**) nonmodified gold electrode and (**b**) Vmh2-ArsC-modified gold electrodes after incubation (1 h, 60 °C) in solutions of 0.5 mM As(III) and transfer in 1 M HCl, range (− 0.4 to + 0.6) V versus Ag/AgCl, scan rate 10 mV s^{-1}. (**c**) SWV of the Vmh2-ArsC-modified gold electrodes after incubation (1 h, 60 °C) in solutions of 0.5 mM As(III) for the (**a**, gray) nonmodified gold electrode, (**b**, dashed line) Vmh2-modified gold electrode and (**c**) 0.1, (**d**) 0.25, (**e**) 0.5, (**f**) 0.75, (**g**) 1, (**h**) 5 and (**i**) 10 mM As(III) for the Vmh2-ArsC-modified gold electrode. SWV parameter: 1 M HCl, pre-deposition at − 0.4 V versus SCE for 5 min, scan rate 100 mV s^{-1}, f = 50 Hz.

metry. *Tt*ArsC has already proven its ability to interact with both arsenate and arsenite[24,25]. Here, owing to the ability of Vmh2-ArsC and ArsC-Vmh2 to strongly interact with gold surfaces, we modified gold electrodes with both Vmh2-ArsC and ArsC-Vmh2. Binding of both arsenate and arsenite were investigated at these enzyme electrodes (Fig. 5a). The biofunctionalized gold was used in a first step to attach arsenic at pH 7.5. Then, the electrode was transferred in a 1 M HCl solution for anodic stripping voltammetry and arsenic detection by Square-Wave voltammetry (SWV). This procedure differs from most of arsenic electrochemical sensors by the fact that ArsC-modified gold electrode is used to attach As(III) to the electrode at pH 7.5, before its detection in 1 M HCl, thus providing an alternative strategy to separate As(III) from the sample before its detection by SWV. In the majority of ASV-based methods As(0) is directly electrodeposited from As(III) sample solution, potentially inducing unwanted stripping peaks and intermetallic deposits[60]. Since gold has no affinity for As(III), either the unmodified gold electrode and the one modified with Vmh2 alone, do not exhibit signals corresponding to arsenic reduction after transfer of the arsenic solution to the 1 M HCl solution (Fig. 5b,c). After soaking the electrode in a 10 mM solution of potassium arsenate and transfer in the 1 M HCl solution, no detection of arsenate reduction into arsenite was observed at gold electrodes. This is mostly due to the fact that As(V) cannot be electrochemically-reduced into As(III) at electrodes[60,61]. The modified gold electrode was also soaked in

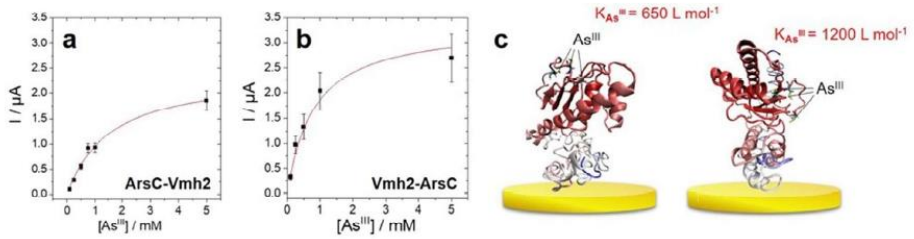

Figure 6. Evolution of the SWV peak current towards starting As(III) concentrations. (**a**) ArsC-Vmh2-modified gold electrode and (**b**) Vmh2-ArsC-modified gold electrodes accompanied by a schematic representation of arsenic binding (**c**). The red line corresponds to the curve fitting using Eq. (2).

a 10 mM solution of sodium (meta)arsenite ($NaAsO_2$). Figure 5b displays the CV of a Vmh2-ArsC-modified electrode in 1 M HCl.

The shape of the CV is indicative of the presence of As(III) at the surface of the electrode (Fig. 5b). This shape is typically observed in anodic stripping voltammetry experiments performed on gold electrodes in acidic As(III) solutions[62–64]. An irreversible reduction wave at − 0.25 V versus Ag/AgCl corresponds to the reduction of As(III) into As(0) which is subsequently oxidized on the reversed scan at + 0.24 V. It is clear that As(III) is immobilized owing to the presence of Vmh2-ArsC and ArsC-Vmh2 at the surface of the electrode. No As(III) was detected on a non-modified gold electrode after soaking the electrode in an As(III) solution. Then, to characterize the interaction between bioelectrodes and arsenite and to examine the ability of the bioelectrode to detect different concentrations of As(III) in water, electrochemical detection was performed at different concentrations using SWV. Figure 5c displays SWV performed after the incubation step (electrodes placed inside solutions with increasing concentrations of arsenic) and the reduction step which fully reduces the attached As(III) into As(0). A shoulder peak might be observed during SWV experiments as observed at 5 mM As(III). This likely arises from the inhomogeneity of As(III) binding on the enzyme layer at higher concentrations. Figure 6 shows the evolution of the peak current towards increasing concentration of arsenic.

Increasing peak current follows a classic Langmuir isotherm model for both immobilized enzymes, which was confidently modelled according to Eq. (2)

$$I_{p,eq} = \frac{I_{p,max} \times K_{As^{III}} \times [As^{III}]}{1 + K_{As^{III}} \times [As^{III}]} \quad (2)$$

where $I_{p,eq}$ is the equilibrium peak current, $I_{p,max}$ is the peak current at saturating concentrations of As^{III} and $K_{As}{}^{III}$ is the association constant between As^{III} and the immobilized enzyme. For ArsC-Vmh2, the best fit was achieved with a $I_{p,max} = 2.5\ (\pm 0.2)\ \mu A$ and $K_{As}{}^{III} = 650\ (\pm 100)\ L\ mol^{-1}$ at 60 °C in 50 mM Tris–HCl pH 7.5 buffer. For Vmh2-ArsC, the best fit was achieved with a $I_{p,max} = 3.4\ (\pm 0.2)\ \mu A$ and $K_{As}{}^{III} = 1200\ (\pm 300)\ L\ mol^{-1}$. Higher $I_{p,max}$ of Vmh2-ArsC indicates that more enzyme is immobilized. This is in good agreement with QCM-D experiments, indicating that Vmh2-ArsC has higher affinity for the surface of gold as compared to ArsC-Vmh2. This might arise from a more accessible Vmh2 domain in the folded Vmh2-ArsC chimera able to interact with surfaces. In addition, higher affinity constant for Vmh2-ArsC might indicate that the *Tt*ArsC domain is more accessible to arsenic binding as compared to immobilized ArsC-Vmh2, confirming the hypothesis that once immobilized Vmh2-ArsC exposes the catalytic moiety better than ArsC-Vmh2. The highly acidic condition of As(III) detection is detrimental to the proteins structure and therefore this kind of biosensor can be used as a single-use sensor. However, owing to the efficient immobilization process and the low amount of the fusion proteins required, the gold electrode surface can be easily re-functionalized.

Conclusions

In this work we developed an electrochemical biosensor based on chimeric proteins endowed with both the adhesive properties of Vmh2, a self-assembling amyloid protein[39] and the arsenic sensing ability of a thermostable arsenate reductase, *Tt*ArsC[13]. Two chimeric genes coding for two alternative fusion proteins (ArsC-Vmh2 and Vmh2-ArsC) were designed, heterologously produced and characterised for their activity and their ability to be immobilized on polystyrene and gold. The results suggest that the position of Vmh2 at the N-terminal of the chimera has a positive effect on its folding, its yield of production (higher for Vmh2-ArsC) and renaturation (higher for Vmh2-ArsC). Furthermore, we observed that the catalytic activity of Vmh2-ArsC is slightly higher than that of ArsC-Vmh2 only when the proteins are immobilized. This result suggests that the expression of Vmh2 at the N-terminal of a chimeric protein[42–44] favors the anchorage on a surface, which improves catalytic activity in terms of substrate accessibility. This is the first time that Vmh2 is expressed at the N-terminal of a chimeric protein[42–44]. Both chimeras demonstrated to be very stable since, once immobilized, they retained their catalytic function for several days and could be reused up to three times; their high stability could be due to the thermophilic origin of *Tt*ArsC and its intrinsic higher resistance to harsh conditions.

arsC-vmh2	
chArsC FW	5′ ATGCGTGTTCTGGTTCTGTG 3′
chVmh2 RV	5′ CAGGCTAATGTTAATCGGGCTG 3′
vmh2-arsC	
chVmh2 FW	5′ ATGGACACCCCGAGCTGTAGCACC 3′
chArsC RV	5′ CAGGCGTGCTGCCTGACGCA 3′

Table 2. Primers employed for PCR colony.

Moreover, the chimeras were used to modify the surface of gold electrodes in order to build an As(III)-sensitive bioelectrode. The results confirm the difference on the substrate accessibility of the immobilized chimeras, which is higher for Vmh2-ArsC. Owing to the interaction of immobilized ArsC and As(III), As(III) was successfully extracted from neutral pH solutions and electroreduced at acidic pHs. Despite the fact that maximum current densities, as well as binding constants have to be increased in order to possibly use these bioelectrodes for As(III) biosensing applications[3,31], this work represents the first example of the use of a thermostable arsenate reductase in an enzyme-based electrochemical arsenic biosensor. The novel use of chimeric enzymes able to provide either a recognition step for As(III) followed by its detection by SWV and an increased ability to bind to hydrophobic surfaces represents an original and promising alternative for arsenic sensing, paving the way to the use of ArsC-Vmh2 and Vmh2-ArsC as a new platform for biosensing in environmental applications. These improvements can be achieved either by further enzyme engineering strategies or by the use of advanced nanostructured electrodes.

Methods

Gene synthesis. Two different gene fusions were designed in order to obtain two versions of the chimeric protein based on the arsenate reductase *Tt*ArsC and the hydrophobin Vmh2; the first (*arsC-vmh2*) presents the gene coding for *Tt*ArsC at 5′end, and the gene coding for Vmh2 at the 3′end; on the other hand, the second (*vmh2-arsC*) presents *vmh2* at the 5′end and *arsC* at the 3′end. Both the synthetic genes were designed to possess a sequence coding for a flexible linker[65] of 15 amino acids between the two proteins and the sequence coding for the thrombin cleavage site (LVPRGS) at the 3′end. The synthetic genes were designed with the GeneArt tool, optimized according to the *E. coli* codon usage and ordered at Thermo Fisher Scientific (https://www.thermofisher.com/it/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis.html).

Bioinformatic analysis. Theoretical isoelectric points and molecular weights of chimeric proteins were determined using the ProtParam tool of ExPASy (https://web.expasy.org/protparam/). Three-dimensional structure models were elaborated using I-TASSER[66] (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the quality of the predicted models was estimated by determining the confidence score (C-score). It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [− 5, 2], where a C-score of a higher value indicates a model with high confidence[46,47]. The 3D-models were visualized using PyMOL v0.99 (https://pymol.org/2/). The propensity to aggregation of chimeras was evaluated on TANGO (http://tango.crg.es/), a computer algorithm for prediction of aggregating regions in unfolded polypeptide chains.

Cloning and heterologous expression. The synthetic genes were cloned in the pET28b(+) expression vectors (Novagen), between *NcoI* and *HindIII* restriction sites, to insert the His-tag at the C-terminal of the resulting proteins. Colonies of *E. coli* Top10F' transformed with pET28b(+)/*arsC-vmh2* and pET28b(+)/*vmh2-arsC* (Supplementary Fig. S3) were screened by PCR colony using the primer pairs chArsC FW, chVmh2 RV for *arsC-vmh2* and chVmh2 FW, chArsC RV for *vmh2-arsC* and Taq DNA Pol (Thermo Scientific) (Table 2). To verify the appropriate insertions, plasmids were recovered from the recombinant colonies using the QIAprep spin Miniprep Kit (QIAGEN) and digested with Nco*I*-HF and Hind*III*-HF (New England Biolabs).

For the protein expression, *E. coli* BL21 (DE3) was transformed with the recombinant vectors. Colonies were grown for 16 h in LB medium containing kanamycin (50 µg/L), and chloramphenicol (33 µg mL/L) then diluted in 1 L of fresh medium with antibiotics. When the culture reached 0.6 $OD_{600\ nm}$, protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the bacterial cultures were grown for additional 6 h at 37 °C.

Purification of chimeras. *Denaturation of inclusion bodies.* Cells were harvested by centrifugation and pellets were resuspended in lysis buffer (Tris–HCl 50 mM pH 8; EDTA 10 mM) and sonicated for 15 min; the sonicator (MISONIX MOD.XL2020) was set up at 35% of amplitude with pulses of 30″ on and 30″ off. The lysate was centrifuged at 15,000 rpm for 60 min (JA25.50 rotor; Beckman). Inclusion bodies were resuspended in Tris–HCl 100 mM pH 8, EDTA 10 mM, Triton X-100 2%, urea 2 M, sonicated for 2 min and centrifugated for 30 min at 15,000 rpm (JA25.50 rotor; Beckman); this step was repeated twice. After 3 washings in Tris–HCl 100 mM pH 8 (preceded each time by 2 min sonication and centrifugation for 30 min), inclusion bodies were resuspended in denaturation buffer (Tris–HCl 50 mM pH 8, Gu-HCl 6 M) and sonicated for further 15 min. Inclusion bodies were then kept under shaking at 37 °C for 16 h after adding β-mercaptoethanol 20 mM.

Conditions	Refolding buffers		Dialysis buffers	
A	A_R	Tris–HCl 100 mM pH 7.5, EDTA 10 mM, NaCl 0.3 M, L-Arginine 0.5 M, DTT 1 mM	A_D	Tris–HCl 50 mM pH 7.5, DTT 1 mM
B	B_R	Sodium-Phosphate 100 mM pH 7.5, EDTA 10 mM, NaCl 0.3 M, L-Arginine 0.5 M, DTT 1 mM	B_D	Sodium-Phosphate 50 mM pH 7.5, DTT 1 mM
C	C_R	Tris–HCl 100 mM pH 7.5, EDTA 10 mM, NaCl 0.3 M, L-Arginine 0.5 M, DTT 1 mM	C_D	Tris–HCl 50 mM pH 7.5, Et-OH 40%, DTT 1 mM

Table 3. List of conditions used to refold ArsC-Vmh2 and Vmh2-ArsC.

Renaturation of chimeras. DTT 2 mM was added to the solubilized inclusion bodies, and incubation prolonged at room temperature for 2 h. The chimeric proteins were refolded by diluting tenfold the solution containing the denatured inclusion bodies solution through addition of renaturation buffer drop by drop. In order to find the best conditions for protein renaturation different refolding buffers were used and they are reported in Table 3. The solutions were then centrifuged for 1 h at 15,000 rpm (JA25.50 rotor; Beckman), and the supernatants were concentrated using an Amicon ultrafiltration system with 3 kDa filters (ULTRACEL Millipore) to get a final concentration of Gu-HCl of 0.15 M and finally dialyzed alternatively against buffer A_D, B_D, or C_D (Table 3).

Analytical methods. Protein concentration of denatured inclusion bodies and of purified ArsC-Vmh2 and Vmh2-ArsC was determined using the Pierce 660 method (Thermo Fischer Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions and using bovine serum albumin (BSA) as the standard.

Protein purification was estimated by SDS-PAGE performed on 12% (w/v) gels supplemented with urea 36%[67]; protein identification was determined by Western blotting performed using (4 µg) of proteins and following standard procedures[68,69]. The detection was performed using monoclonal anti-polyHistidine antibody (1:10,000) (Sigma-Aldrich), and chemiluminescent reaction with the "Immobilon Western chemiluminescent horseradish peroxidase (HRP)" kit (Millipore). Acquisitions were developed with ChemiDoc XRS (Bio-Rad).

Immobilization on polystyrene plate. 100 µL of ArsC-Vmh2 and Vmh2-ArsC at different concentrations (0.025; 0.05; 0.1; 0.2 mg/mL) were deposited into the wells of polystyrene multi-well plates (96 wells) and incubated at 28 °C for 16 h. Controls were obtained by depositing 100 µL of renaturation buffer A (50 mM Tris–HCl pH 7.5, 0.15 M GuHCl, 1 mM DTT) or 100 µL of purified *Tt*ArsC at the above indicated concentrations. After incubation, to eliminate unbound proteins, the wells were washed three times with 100 µL of 50 mM Tris–HCl, pH 7.5. The amount of immobilized proteins was calculated by subtracting the amount of unbound proteins (determined through the Pierce 660 method) and the immobilization yield was calculated as the ratio (%) between µg of immobilized proteins and µg of proteins initially deposited into the wells. All the experiments were performed in triplicates. Statistical analysis was performed through the ordinary one-way ANOVA on GraphPad Prism 7.00; significant differences of TtArsC in comparison to chimeras are indicated as: $*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$.

$$Immobilized\ proteins = Deposited\ proteins - Unbound\ proteins$$

$$Yield\ of\ immobilization = \frac{Immobilized\ proteins}{Deposited\ proteins}\%$$

Arsenate reductase activity assay. Arsenate reductase activity was measured using a coupled assay that follows the arsenate dependent oxidation of NADPH in the presence of Trx from *E. coli* and Tr from *Sulfolobus solfataricus* (*Ss*Tr) both purified following reported procedures[55,56]. A typical assay was performed in a final reaction volume of 160 µL at 60 °C in a double beam spectrophotometer (Cary 100, Varian). The arsenate reduction activity was measured by following the decrease in absorption at 340 nm due to the oxidation of NADPH[12] as described by Del Giudice et al.[13]. One unit of enzyme activity (U) was defined as the amount of enzyme required to consume 1 µmol NADPH per minute, under the assay condition described. The specific activity is reported as units of enzyme activity per milligram of enzyme (U/mg). Each reaction was performed in triplicate, and in the same buffer condition.

Phosphatase activity assay. The phosphatase activity of chimeras and of chimeras immobilized in polystyrene plates was measured at 60 °C using pNPP as substrate (Sigma-Aldrich) and following the increase in absorption at 405 nm due to the formation of *p*-nitrophenol ($\Delta\varepsilon 405 = 18{,}000\ M^{-1}\ cm^{-1}$)[13]. Each reaction was performed in triplicate in a plate reader spectrophotometer (Sinergy H4, software version 2.07.17) in a total volume of 160 µL containing 4 µM of ArsC-Vmh2 or Vmh2-ArsC, 60 mM pNPP in 50 mM Tris–HCl pH 7.0. As controls the same reactions were performed: (a) in the absence of enzyme, (b) with immobilized *Tt*ArsC (4 µM). The variation of Abs_{405nm} per minute (ΔOD/min) obtained in the absence of enzyme was subtracted to that one obtained from the experiments with enzymes. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol *p*-nitrophenol *per* minute, under the assay condition described. The specific activity of immobilized proteins was calculated as enzyme units per milligram of proteins adhered to polystyrene as determined above (Immobilization on polystyrene plate).

Quartz-crystal microbalance analysis with dissipation monitoring (QCM-D). QCM-D measurements were performed using E1 instruments (Q-Sense, AB, Göteborg, Sweden) equipped with one laminar flow chambers and polished AT-cut piezoelectric quartz crystals (diam. 14 mm) covered by a 100 nm thick gold layer (QSX 301-Q-Sense). f and D were measured at the fundamental resonance frequency (5 MHz) as well as at the third, fifth, seventh, ninth, eleventh, and thirteenth overtones (n = 3, 5, 7, 9, 11 and 13). Experiments were conducted in a continuous flow at a flow rate of 50 µL min^{-1}. For each experiment the instrument was equilibrated with 50 mM Tris–HCl pH 7.5, 0.15 M Gu-HCl, 1 mM DTT buffer. After 15 min of signal stabilization, 500 µL of the enzyme solution (0.04 mg mL^{-1} in buffer 50 mM Tris–HCl pH 7.5, 0.15 M Gu-HCl, 1 mM DTT) was injected during 10 min (50 µL/min) and then the flow stopped for 20 min before flowing the buffer solution to remove the unbound enzyme, until signal stabilization. Each set of experiment was performed in duplicate.

Electrochemical analysis. The electrochemical experiments were carried out in a three electrodes electrochemical cell using a Biologic VMP3 Multi Potentiostat. Gold electrodes (0.071 cm^2) were used as working electrodes. Pt wire was used as counter electrode and a silver chloride electrode (Ag/AgCl) served as reference electrode. All potentials are given versus Ag/AgCl.

To immobilize ArsC-Vmh2 and Vmh2-ArsC on gold electrodes 20 µL of chimeric enzymes (0.2 mg mL^{-1}) were deposited on the electrode surfaces, and dried at 4 °C overnight. As negative controls, *Tt*ArsC and Vmh2 were used. The electrodes were then washed with 50 mM Tris–HCl pH 7.5 and incubated for 1 h at 60 °C in different solutions of sodium (meta)arsenite ($NaAsO_2$) ranging from 0.1 to 5 mM. After incubation, Cyclic voltammetry (CV) and Square Wave Voltammetry (SWV) experiments were performed in 1 M HCl solution. Each set of experiment was performed in triplicate.

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Author contributions
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Phosphatase activity inhibition of the arsenate reductase *Tt*ArsC from *Thermus thermophilus*: its potential application as arsenic biosensor

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*PAPER IN SUBMISSION

Abstract

Arsenic (As) pollution is a widespread problem worldwide. The World Health Organization (WHO) classifies arsenic among the 10 chemicals of major public health concern. In last years, many biosensors for arsenic detection have been developed, most of them based on enzymatic inhibitions: inhibition-based biosensors. The principle of this type of biosensors is the quantification of the inhibitor (i.e. the arsenic), by the measure of the enzymatic activity in its absence and presence. Therefore, the study of the enzymatic inhibition is a key point for the development of an inhibition-based biosensor. The arsenate reductase *Tt*ArsC from *Thermus thermophilus* HB27 converts As(V) into As(III), but it is also provided with a secondary phosphatase activity. In this work we investigate the interference of As(V) and As(III) on this phosphatase activity through a colorimetric assay, using p-nitrophenylphosphate (pNPP) as substrate. In particular, Michaelis-Menten kinetics and Lineweaver–Burk double-reciprocal plots show that both As(V) and As(III) are non-competitive inhibitors, affecting the V_{max} (19.29 ± 1.14 nmol/min/mg), but not the K_M (9.57 ± 1.95 mM) of the reaction. However, their Ki values are very different 15.2 ± 1.6 µM for As(V) and 394.4 ± 40.3 µM with As(III), indicating the major affinity of the enzyme to the first. The potential application of *Tt*ArsC as inhibition-based biosensor is investigated by the construction of an As(V) dose-response curve: the system exhibits a sensitivity of 0.53 ± 0.03 mU/mg/µM and a LOD of 0.28 ± 0.02 µM, and it is also possible observing the complete inhibition of the reaction with naked eye at 50 µM of As(V). Moreover, testing this system on other ions, toxic compounds, and in saline solutions, the *Tt*ArsC phosphatase activity is mainly not affected, highlighting the specificity to As(V) and the real

possibility of exploiting *Tt*ArsC phosphatase activity for the development of an As(V) inhibition-based biosensor.

Introduction

Arsenic is a toxic metalloid, commonly occurring as a groundwater pollutant. It largely derives from mines, industrial wastes or geochemical processes and both its organic and inorganic forms can be found in the environment [1]. Among polluting inorganic arsenic species, arsenate As(V) and arsenite As(III) are the most common and toxic forms; in particular As(III) is almost 60 times more toxic than As(V), but this last is the predominant form in environment, therefore, the monitoring of both of them is necessary [2]. Arsenic is one of WHO's 10 chemicals of major public health concern: millions of people all over the world are exposed to arsenic concentrations much higher than the guideline value (10 µg/L in drinking water) and the effects of long-term exposures at toxic concentrations are the cause of many diseases that in extreme cases lead to death. For these reasons, in the 2030 Agenda for Sustainable Development, the indicator of "safely managed drinking water services" is aimed to guarantee to all people the access to drinking water, free of microbial and chemical contaminants, including arsenic [3–5].

The traditional approaches for monitoring the arsenic pollution are based on chemical or physical analysis and allow highly accurate and sensitive determination of the exact composition of any sample. These analyses require specialized operators and expensive instrumentations, as the ICP-MS (Inductively Coupled Plasma - Mass Spectrometry) which is the most adopted technique for detecting heavy metals, to date [6]. Furthermore, also ICP-MS methodologies need to be implemented with other techniques, as high-performance liquid chromatography (HPLC), if information about the arsenic speciation in the samples is desired [7]. The need for accurate, but less expensive measurements has led to the development of sensors based on biomolecules: biosensors. These analytical devices integrate a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of the analyte [8]. Thanks to the biological component, biosensors can also provide an alternative to toxicity measurements, with respect to the classical approaches [9].

In recent years, many biosensors for arsenic detection have been developed, but despite all the promising advancements and demonstrations of proof of principles, there are very few studies that

have critically evaluated arsenic bioassays under real life field conditions [10]. These biosensors present as biological component either whole-cell or biomolecules like aptamer, DNA, and enzymes [11]. Whole-cell biosensors have the advantages of specificity, low cost, ease of use, portability and continuous real-time signals emissions. However, their use is limited by long incubation periods and too high detection limits [12,13].

Cell-free biosensors overcome these issues; in particular enzyme biosensors have a fast response time, but may undergo interferences with other ions, i.e. they lack of specificity [10,14]. In recent years many arsenic biosensors have been developed that measure arsenic through enzymatic inhibition. Such inhibition biosensors quantify the analyte measuring its concentration-dependent effect on the selected enzymatic activity. Inhibition biosensors are of interest in clinical field to quantify drugs that inhibit enzymes of specific biological pathways; moreover, biosensors based on enzyme inhibition have been used to detect toxic compounds in food and environmental samples [17,18]. For example, Sanllorente and co-workers developed electrochemical biosensors both for As(III) and As(V), the first based on the acetylcholine inhibition, the latter on that of an acid phosphatase [15,16]; these biosensors, tested under laboratory conditions, have detection limits in the range of permissible exposure limit for arsenic, but their application is often compromised by the presence of other ions in the water sample or in the field [10–13]. In fact, to date, the commercialization of biosensors based on enzyme inhibition is still untimely [18].

Another bottleneck in the development of enzyme-based biosensors is represented by enzyme stability [19]. To overcome this drawback, engineered enzymes [20,21], novel surface nanostructured biosystems [22], or enzymes immobilized on nanomaterials [23,24] have been investigated. Increasing attention has been also put on the employment of enzymes from extremophiles, commonly considered as sources of stable biomolecules [25,26]. For example, thermozymes belonging to thermophilic microorganisms are able not only to thrive at high temperatures, but to several harsh conditions as for example high concentrations of heavy metals, salts and low pH [27–30]. In this context, *Tt*ArsC, an arsenate reductase from the thermophilic bacterium *Thermus thermophilus* HB27 [31], has been exploited to set up arsenic biosensors, thanks to its ability to react with As(V) [20,32,33]. *Tt*ArsC was discovered as a component of the arsenic resistance system of *T. thermophilus* HB27; in the resistance system *Tt*ArsC is the very thermostable enzyme able to reduce As(V) to As(III); As(III) that is more

toxic for the cells is actively extruded by a membrane P_{1B} type ATPase, named *Tt*ArsX [31,34]; either *Tt*ArsC and *Tt*ArsX are regulated at transcriptional level by the metal responsive transcriptional repressor *Tt*SmtB [35,36].
*Tt*ArsC belongs to the family of thioredoxin-coupled arsenate reductases, whose paramount enzyme is ArsC from *Staphylococcus aureus* plasmid pI258 [37]. Members of this family are characterised by the presence of three redox active cysteines, one (Cys7 in *Tt*ArsC) performing the nucleophilic attack to the substrate and the other two (Cys 83 and Cys 90 in *Tt*ArsC) forming a disulphide bond following the reduction of As(V) into As(III) [37]. To regenerate the enzyme reducing equivalents flow through NADPH- thioredoxin reductase (Tr)-thioredoxin (Trx) system, reducing the disulphide bond [37]. Within the family, the first cysteine residue is encompassed in a conserved CX_5R motif, known as P-loop; this motif and the conserved Asp-Pro sequence (Asp105 - Pro106) are also found in low molecular weight protein tyrosine phosphatases (LMW PTPase) and confer phosphatase activity to many thioredoxin-coupled arsenate reductases [38]. In fact, it has been proposed that arsenate reductase activity evolved from LMW PTPase by a change of mechanism, that maintained the oxyanion substrate binding and performed phosphatase activity using amino acids of the P-loop [37,38]. *Tt*ArsC was endowed with phosphatase activity but it was much weaker than that of ArsC from *S. aureus* pI258, probably because of differences in the amino acid composition of the active-site loop [31].
Considering that biosensors, and in particular those based on enzymatic inhibitions, are useful as general toxicity indicators for the fast identification of contaminated samples [39], in this work we analyze the effect of arsenic on *Tt*ArsC phosphatase activity, with the aim to evaluate its exploitation as an arsenic enzymatic inhibition-based biosensor. Moreover, in reason of what discussed above about the specificity in arsenic biosensors, we also investigate the interference of other ions and toxic compounds on *Tt*ArsC phosphatase activity.

Materials and Methods

-Chemicals

The metal salts used in this work were purchased by Sigma-Aldrich and they are: Sodium (meta) arsenite ($NaAsO_2$); Sodium arsenate dibasic heptahydrate ($Na_2HAsO4 \cdot 7H2O$); Cadmium chloride ($CdCl_2$); Cobalt chloride ($CoCl_3$); Copper chloride ($CuCl_2$); Mercury chloride, ($HgCl_2$); Nickel chloride ($NiCl_2$). Also the substrate (pNPP) 4-Nitrophenyl

phosphate disodium salt hexahydrate (CAS Number 333338-18-4) and all the pesticides were purchased from Sigma-Aldrich: malathion (CAS number: 121-75-5), parathion (CAS Number: 56-38-2), fenitrothion (CAS Number: 122-14-5) and paraoxon-ethyl (CAS Number: 311-45-5).

-Phosphatase activity assay

The phosphatase activity of *Tt*ArsC was measured at 60 °C using pNPP as substrate (Sigma-Aldrich) and following the increase in absorption at 405 nm for 1h, due to the formation of p-nitrophenol (ε_{405} = 18,000 M^{-1} cm^{-1}). Each reaction was performed in triplicate in a plate reader spectrophotometer (Sinergy H4, software version 2.07.17) in a total volume of 160 µL containing 4 µM of enzyme in 20 mM Tris-HCl pH 7.00. Substrate concentrations used ranged from 0.25 to 200 mM (see below). As negative control, the same reactions were performed in the absence of enzyme (blank control). The variation of Abs_{405nm} per minute (ΔOD/min) obtained in the absence of enzyme was subtracted to that obtained from the experiments with enzymes. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol p-nitrophenol per minute, under the above assay condition, therefore 1 nmol/min/mg corresponds to 1 mU/mg.

-Inhibition assays

The effect of As(V) and As(III) on phosphatase activity was determined measuring the phosphatase activity in the presence of fixed concentrations of metal ions at varying pNPP substrate concentration. Michaelis and Menten curves were obtained measuring the enzyme activity in the presence of: 0 – 1 – 5 – 10 – 20 µM or 0 – 50 – 100 – 150 – 500 µM, As(V) and As(III), respectively. For each curve the pNPP substrate concentrations were: 0.25 – 0.5 – 1 – 2 – 4 – 8 – 10 – 15 – 20 – 30 – 40 – 100 – 200 mM. All the experimental sets were performed in triplicate.

As(V) dose-response curve

The As(V) dose-response curve was obtained measuring the enzyme activity using pNPP 40 mM and As(V) at final concentrations of 0 – 1 – 5 – 10 – 20 – 30 – 40 – 50 – 60 – 70 – 80 – 90 – 100 µM. As negative control, the same reaction was performed in the absence of enzyme and inhibitor (blank control). The experiments were performed in triplicate.

Pesticides inhibition

Pesticides were tested as inhibitors at two different pNPP concentration: 2 and 40 mM. For each assay malathion, parathion, fenitrothion or paraoxon-ethyl were added to reaction to final concentrations of 50, 100, 200, 300 or 500 µM and the enzyme assay performed as described above. The experiments were performed in triplicate.

Metals inhibition

The phosphatase activity of *Tt*ArsC in presence of other heavy metals was also investigated. Cd(II), Hg(II), Ni(II), Co(II) and Cu(II) 10, 50, or 100 µM were added to reactions performed at saturating concentration of pNPP (40 mM); the residual activity was evaluated in comparison to the activity without metals, considered as 100%. The experiments were performed in triplicate.

Moreover, to evaluate whether the presence of these metals affected the determination of the As(V) concentration, As(III), Cd(II), Hg(II), Ni(II), Co(II) and Cu(II) were added to reactions at final concentrations of 10 – 50 and 100 µM in presence of a fixed As(V) concentration (10 µM). Experiments were performed using saturating pNPP concentration (40 mM). The percentage of retained activity in the presence of metals was evaluated in comparison to the activity without metals, considered as 100%. The experiments were performed in triplicate.

Complex saline solutions inhibition

*Tt*ArsC phosphatase activity assay was also tested in complex saline solutions, substituting the volume of ddH_2O in the reaction (~130 µL) with commercial drinking waters or a culture medium (BAT-HIMEDIA). Experiments were performed with saturating concentrations of pNPP (40 mM). Moreover, to evaluate the interference of these waters on the As(V) detection, As(V) was added in solutions at final concentrations of 5 and 10 µM.

The composition of commercial drinkable waters is reported in Table 1 (the salts composition reported on the labels of the tested drinking waters) and the composition of the culture medium (BAT-HIMEDIA) is available at the web-site https://himedialabs.com/TD/M1561.pdf .

Table 1. Salts composition reported in drinkable waters (DW). All the values are considered as mg per liter of water. N.R. = Not Reported.

Salts		DW1 (mg/L)	DW2 (mg/L)	DW3 (mg/L)
Bicarbonate	HCO_3^-	321	215.0	498
Calcium	Ca^{2+}	87.0	71.0	124
Magnesium	Mg^{2+}	17.6	5.5	29.8
Silica	SiO_2	8.0	16.6	N.R.

Nitrate	NO_3	3	9.4	2
Sodium	Na^+	4.9	11.7	4.0
Sulphates	SO_4^{2-}	25.6	10.7	17.6
Chlorides	Cl^-	7.7	17.9	6.6
Potassium	K^+	1.2	1.2	1.2
Fluorides	F^-	<0.2	<0.10	N.R.
Ammonium	NH_4^+	N.R.	<0.05	N.R.
Nitrites	NO_2^-	N.R.	<0.002	<0.002

-Data Analysis

All the experiments were performed in triplicates. Data were analysed using GraphPad Prism 7.00 [40] and statistical analyses were performed through the ordinary one-way ANOVA; significant differences in *Tt*ArsC phosphatase activities are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results and Discussions

-Arsenic Inhibition profile

Phosphatase activity of *Tt*ArsC was detected through a colorimetric assay, using p-nitrophenylphosphate (pNPP) as substrate. Michaelis-Menten kinetics and Lineweaver–Burk double-reciprocal plots showed for this enzyme a K_{cat} of 0.005 s^- and a K_M of 9.57 ± 1.95 mM, with a R^2 value of 0.9571. Then, the inhibitory effect of different concentrations of As(V) and As(III) was determined. (**Figure 1**). The R^2 values for individual Michaelis–Menten fittings are reported in **Table 2** and **Table 3**, respectively for As(V) and As(III).

As(V) inhibition

The increase in concentrations of As(V), from 0 to 20 µM, resulted in a V_{max} decreasing from approximately 19.29 ± 1.14 to 6.14 ± 0.33 nmol/min/mg (**Figure 1a, Table 2**). On the other hand the K_M constant resulted almost unvaried, indicative of a noncompetitive inhibition mechanism [41,42]. Lineweaver–Burk double-reciprocal plots also indicated a noncompetitive inhibition, since the convergence of related data sets on the X-axis was observed. This result was also confirmed through the kinetics fitting by nonlinear regression: the noncompetitive inhibition model fit by nonlinear regression yielded overall a R^2 value of 0.9499.

As(III) inhibition

Also at increasing concentrations of As(III) we observed a V_{max} decreasing that changed from approximately 19.29 ± 1.14 to 7.79 ± 0.43 nmol/min/mg and an almost unvaried K_M. However, the inhibitory

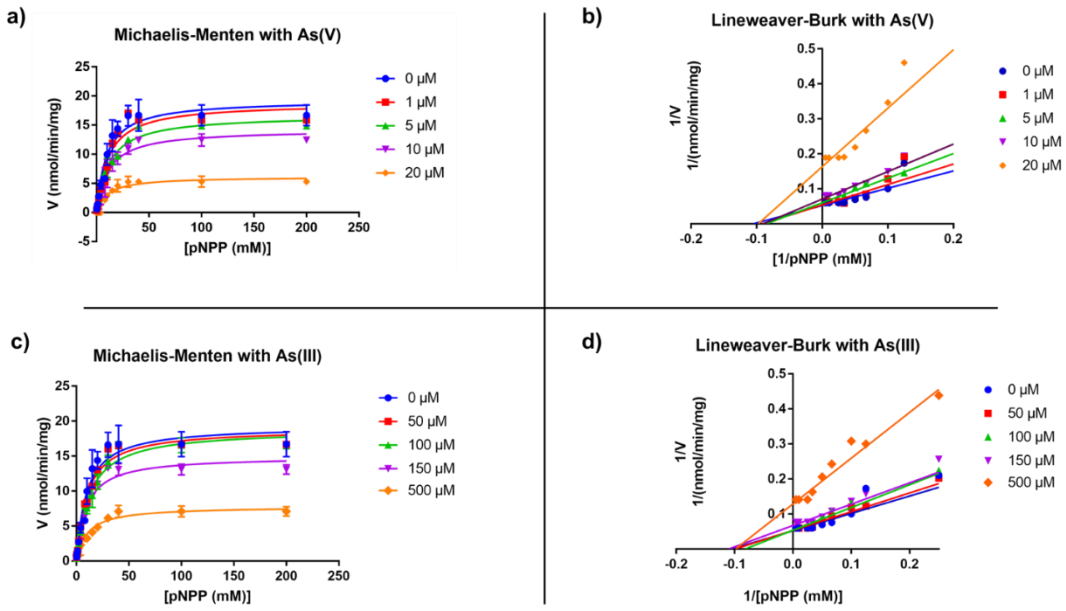

Figure 5. Graphical representation of Michaelis-Menten kinetics at different concentrations of **a)** As(V) and **c)** A(III) and their relatives Lineweaver-Burk double reciprocal plots in presence of **b)** As(V) and **d)** As(III).

effect was obtained at much higher As(III) concentration (**Figure 1c and Table 3**). Also in this case the Lineweaver–Burk double-reciprocal plot was evaluated indicating a noncompetitive inhibition, confirmed by the fitting by nonlinear regression, which exhibited a R^2 value 0.9633 to the noncompetitive inhibition model.

Table 2. Kinetics parameters, V_{max} and K_M, of the *Tt*ArsC phosphatase activity and respective R^2 values for nonlinear regression fitting at different concentration of As(V).

As(V) (µM)	V_{max} (nmol/min/mg)	K_M (mM)	R^2
0	19.29 ± 1.14	9.57 ± 1.95	0.9571
1	18.77 ± 1.36	11.00 ± 2.65	0.9429
5	16.70 ± 0.64	11.75 ± 1.46	0.9827
10	14.22 ± 0.58	11.18 ± 1.52	0.9807
20	6.14 ± 0.33	10.27 ± 1.86	0.9660

Table 3. Kinetics parameters, V_{max} and K_M, of the *Tt*ArsC phosphatase activity and respective R^2 values for nonlinear regression fitting at different concentration of As(III).

As(III) (μM)	V_{max} (nmol/min/mg)	K_M (mM)	R^2
0	19.29 ± 1.14	9.57 ± 1.95	0.9571
50	18.91 ± 0.74	10.13 ± 1.34	0.9810
100	18.79 ± 0.77	12.24 ± 1.61	0.9811
150	14.95 ± 0.65	9.11 ± 1.39	0.9753
500	7.79 ± 0.43	10.20 ± 1.89	0.9552

Since *Tt*ArsC exhibited a noncompetitive inhibition profile with both As(V) and As(III), the Ki values were determined through a nonlinear regression fit according to noncompetitive inhibition models, resulting in values of 15.2 ± 1.6 μM for As(V) and 394.4 ± 40.3 μM for As(III). Therefore, the affinity of *Tt*ArsC towards As(V) is almost 20 times greater than that of As(III). Despite the models suggest a noncompetivite inhibition both for As(III) and As(V), the higher affinity of this latter could be due to the fact that As(V) is the natural substrate of *Tt*ArsC. In *Tt*ArsC the catalytic nucleophile Cys7 is also the first aminoacidic residue of the P-loop, therefore it could be involved in both phosphate and arsenate binding, while the other two conserved Cys residues (Cys82 and Cys89), essential for the reduction of the As(V) into As(III), are spatially separated from the P-loop [31]. As fact, Messens and coworkers demonstrated through NMR analysis that both arsenate and phosphate ions bind the first Cys residue of the P-loop of ArsC from *S. aureus* pI258 [38].

-As(V) dose-response inhibition

Considering that *Tt*ArsC inhibition is deterined in a lower concentration range of As(V) with respect to As(III), we decided to investigate the response of *Tt*ArsC to As(V) by constructing an As(V) dose-response curve. The assays were performed using 40 mM of pNPP (concentration at Michalis-Menten plateau) and varying As(V) concentration. The values of *Tt*ArsC specific activity in function of As(V) were fitted to the classical model: [Inhibitor] vs. response - Variable slope [40] (**Figure 2a**). This model allows to determine the IC50 of the inhibitor, i.e. the concentration that provokes a response half-way between the maximal (Top) response and the maximally inhibited (Bottom) response. Figure **2a** shows that increasing concentration of As(V) determines an inhibition profile with good confidence (R^2 = 0.9959) described by the equation (1):

(1) $$y = B + \frac{(T-B)}{1+\frac{x^{HillSlope}}{IC50^{HillSlope}}}$$

Where **T** (Top response) and **B** (Bottom response), are respectively 16.20 ± 0.32 and 0.59 ± 0.24 nmol/min/mg; **IC50** is 13.16 ± 0.63 µM and the **HillSlope**, which describes the steepness of the family of curves, is 2.60 ± 0.26. The equation allows to calculate the incognito concentration of As(V) in solution.

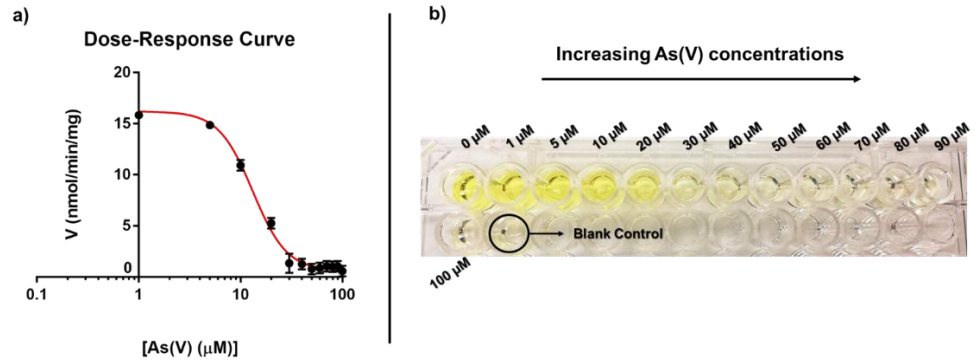

Figure 6. a) Dose-response curve of the inhibition of *Tt*ArsC phosphatase activity at increasing concentration of As(V), reported in panel b). **b)** *Tt*ArsC phosphatase activity assay in multiwell plate at increasing concentrations of As(V).

As shown in **Figure 2b**, at As(V) concentrations higher than 50 µM the reaction is completely inhibited since the solution appears colourless. Therefore, if As(V) crosses this concentration it is possible a naked eye evaluation of the inhibition. Moreover, we calculated the limit of detection (LOD) according to the 3sd/m criterion, where m is the sensitivity, i.e. the slope of the curve in its linear range and sd is the standard deviation of the blank [43,44]. The inhibition system exhibited a sensitivity of 0.53 ± 0.03 mU/mg/µM and a LOD of 0.28 ± 0.02 µM. These results due to the inhibition of *Tt*ArsC phosphatase activity by As(V) are in agreement with other characterized As(V) inhibition based biosensors [45,46], among which the best performances were achieved by an amperometric AcP biosensor, presenting a LOD of 0.11 µM in a linear range of 0.1 to 1.3 µM of As(V), that was also tested on ground water samples [16]. Other examples of biosensors employing an enzymatic inhibition provoked by As(V), include a laccase self-powered biosensor showing LOD of 132 µM and sensitivity 0.98 ± 0.02 mV/mM [47] and an acid phosphatase-polyphenol oxidase biosensor presenting a LOD of 2 nM in a As(V) linear range of 8.9 to 79 nM [48]; both these were also tested against As(III): the first exhibit LOD of 13 uM and

sensitivity of 0.91 ± 0.07 mV/mM [47], while the latter is more specific since does not suffer interferences by As(III) [48].
In our case, we already knew that the affinity of *Tt*ArsC towards As(V) is 20 times higher with respect to As(III) (see above), but the following experiments were aimed to evaluate the interference of As(III) and the applicative potential of *Tt*ArsC inhibition system for the As(V) detection on more complex solutions.

-Other metals

The interference of As(III) and other heavy metals on the phosphatase activity was also investigated. In particular, we evaluated the residual phosphatase activity after addition to the enzymatic mix of As(III), Cd(II), Hg(II), Ni(II), Co(II) or Cu(II) at three different concentrations (10, 50 or 100 µM), considering as 100% the activity in the absence of metals. The effect was compared with that of As(V). As shown in **Figure 3a** significative inhibition of phosphatase activity was observed in the presence of Co(II) and Cu(II); on the other hand, the activity measured with As(III), Cd(II), Hg(II) and Ni(II) was similar to the control without metals, suggesting that these metals were not inhibitory.
Since no effect on activity was observed with the majority of metal ions tested, we asked whether the specific inhibitory effect of As(V) was affected by the co-presence of heavy metals. Therefore, the phosphatase activity was tested at a fixed concentration of As(V) (10 µM) close to the IC50 (see above), adding separately the other metal ions (**Figure 3b**). As can be seen form the figure, with the exception of Co(II) and Cu(II), the presence of As(III), Cd(II), Hg(II) and Ni(II) does not alter to a significant extent the inhibitory effect of 10 µM of As(V); indeed it provokes a residual phosphatase activity of ~74%, which by the addition of Co(II) and Cu(II) decreases to ~30% and ~21%.

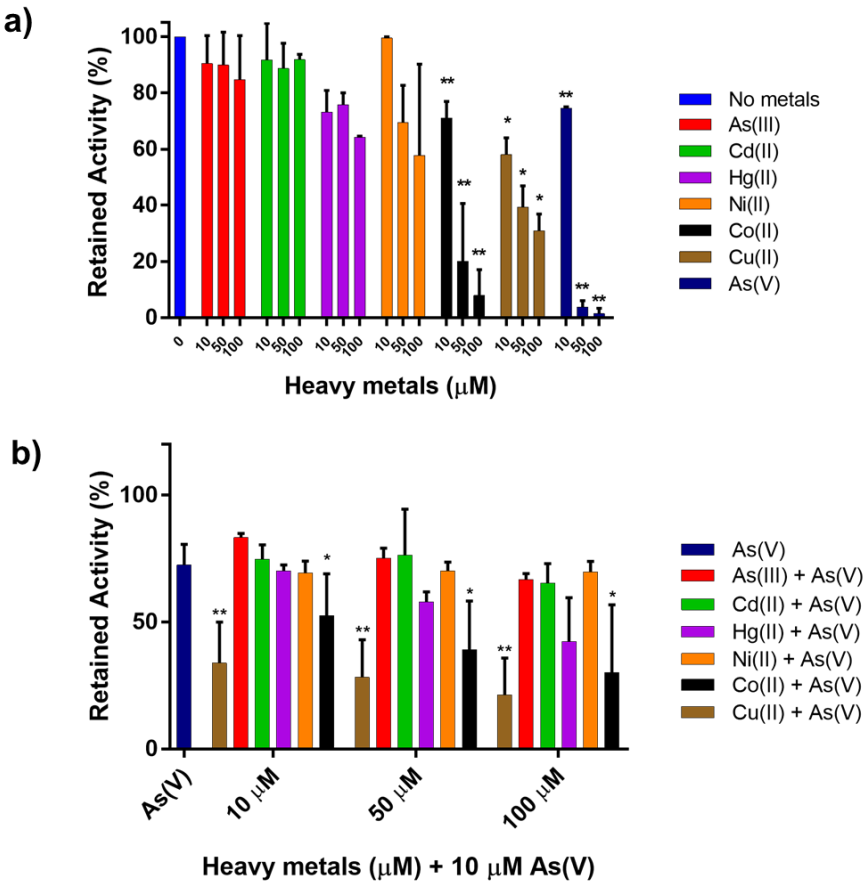

Figure 3. a) Retained activity of *Tt*ArsC in presence of 10, 50 and 100 μM of heavy metals; **b)** Retained activity of *Tt*ArsC in presence of a fixed concentration of As(V) (10 μM) and increasing concentrations of other heavy metals (10, 50 and 100 μM). Heavy metals are reported as following: As(V) in dark blue, As(III) in red, Cd(II) in green, Hg(II) in purple, Ni(II) in orange, Co(II) in black, Cu(II) in brown. Statistical analysis was performed through the ordinary one-way ANOVA on GraphPad Prism 7.00; significant differences with respect to **a)** *Tt*ArsC activity without metals and **b)** to *Tt*ArsC activity in presence of As(V) (10 μM) are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Pesticides

It is well known that organophosphate pesticides inhibit many enzymatic activities; among these, alkaline phosphatases, that have been investigated as possible pesticides biosensors, thanks to the structural similarity of these molecules to phosphate-substrates [19,49–51].

With the aim to evaluate if the phosphatase activity of *Tt*ArsC was affected by these molecules, we performed experiments in the

presence of increasing concentrations of malathion, parathion, fenitrothion and paraoxon-ethyl. These are among the most toxic and used pesticides in the agricultural field, and their monitoring in environment is an urgent need [52,53]. When the assays were performed using saturating pNPP concentration, no variation in the phosphatase activity of *Tt*ArsC was observed by adding the pesticides (data not shown), probably because as substrate analogs they could provoke a competitive inhibition [54,55]. Therefore, we decided to analyse *Tt*ArsC activity using 2 mM of pNPP, a value well below its K_M (9.57 ± 1.95 mM) and closer to the concentrations of pesticides we tested. In this way, we analyzed the behavior of the enzyme in conditions of full availability of substrate binding sites. Interestingly, we found a linear decrease of *Tt*ArsC specific activity with increasing concentrations of malathion, parathion and paraoxon-ethyl and no effect by fenitrothion (**Figure 4**). These results indicate that though some organophosphate pesticides are able to inhibit the phosphatase activity of *Tt*ArsC, the required conditions are not those we investigated for the set-up of the As(V) biosensing strategy, which actually provide for saturating concentrations of pNPP.

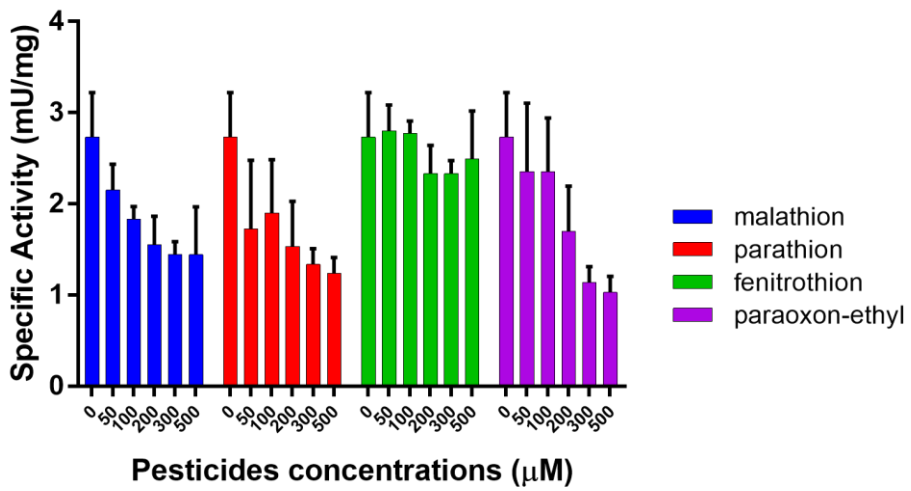

Figure 4. Specific activity of *Tt*ArsC with 2 mM of pNPP, in presence of 50, 100, 200, 300 and 500 µM of pesticides. Pesticides are reported as following: malathion in blue, parathion in red, fenitrothion in green, paraoxon-ethyl in purple.

-Aqueous saline solutions

Since data obtained with other potential pollutants have shown that the majority of those tested does not interfere with the inhibition by As(V)

of *Tt*Arsc phosphatase activity, we wondered if more complex solutions in which more metals are present at the same time could have effects. Therefore, we decided to evaluate the phosphatase activity using drinking waters and a bacterial culture medium. Drinking waters contain magnesium, calcium, sodium, and potassium salts, as well as bicarbonates, sulfates, nitrates and nitrites in different concentrations and the chosen culture media at pH 4, contains several salts and other metals in micro-traces (BAT medium [56]).
The results show that also in presence of these more complex solutions, *Tt*ArsC retains its specific phosphatase activity. In order to assess whether under these conditions *Tt*ArsC was still inhibited by As(V) in the same way, we repeated the test in the presence of 5 and 10 µM of As(V) and the results are shown in **Figure 5**. Since no significative differences were highlighted by the comparison with reactions performed in ddH_2O we concluded that the inhibitory effect dose-response of As(V) on *Tt*ArsC is specific also in more complex solutions. Therefore, these results let us to speculate on a potential exploitation of this system on real water samples.

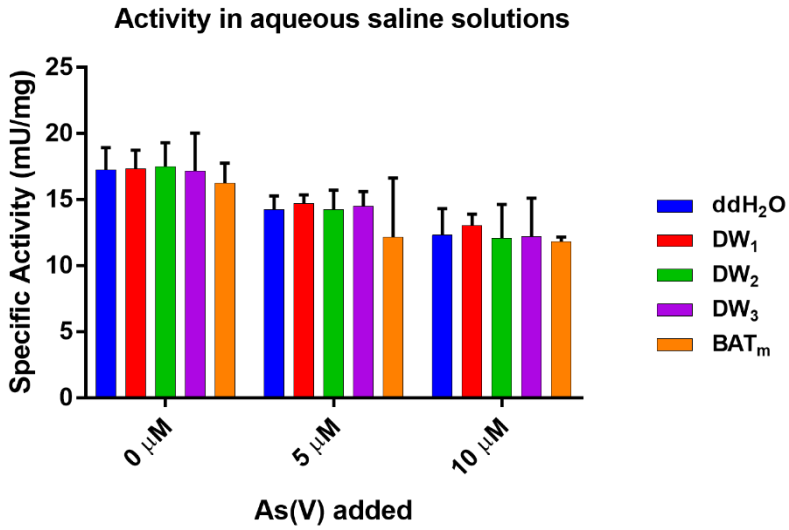

Figure 5. *Tt*ArsC phosphatase activity performed in ddH_2O, three drinkable waters (DW) and in a culture medium (BAT_m). As control, As(V) was added at final concentrations of 5 and 10 µM.

Conclusions

The phosphatase activity of the thermophilic arsenate reductase *Tt*ArsC is a secondary activity of this enzyme that does not need the intervention of the redox cascade system Trx-TrxR, required for the

conversion of As(V) into As(III). This activity can be easily measured following the release of p-nitrophenol at 60 °C. In this paper, we investigated the effect of arsenic and other different toxic contaminants, on the phosphatase activity in order to verify a potential employment of *Tt*ArsC as an arsenic optical biosensor.

We found that both As(V) and As(III) behave as non-competitive inhibitors affecting V_{max} which decreases from 19.29 ± 1.14 to 6.14 ± 0.33 and 7.79 ± 0.43 nmol/min/mg respectively, but not K_M (9.57 ± 1.95 mM). However, the Ki value determined with the substrate of the reductase activity, i.e. As(V) (15.2 ± 1.6 µM) is almost 25 times lower than that determined with As(III) (394.4 ± 40.3 µM).

Therefore, we further investigated the system for the development of an As(V) biosensor, by the construction of dose-response curve.

Our results show that under the value of As(V) 50 µM the system exhibits a sensitivity of 0.53 ± 0.03 mU/mg/µM and a LOD of 0.28 ± 0.02 µM revealed by the use of a spectrophotometer. Moreover, it is possible to see the complete inhibition of the reaction with naked eye from 50 µM of As(V) onwards. This result indicates that within a micromolar range of As(V) concentration, a complete inhibition of the phosphatase activity is achieved, laying the basis for potential naked eye evaluations of large amounts of As(V) in solution. Interestingly, almost all the other heavy metals that we tested (except for Co(II) and Cu(II)), as well as organophosphate pesticides caused enzyme inhibition at higher concentration that are in a millimolar range. This result suggests that a biosensor based on the inhibition of *Tt*ArsC phosphatase activity is not affected by the presence of such ions and/or molecules in that range. In addition, the data obtained by testing phosphatase activity in more complex solutions shows that the system is effective in detecting As(V) even under such conditions. All together our results let us to speculate that the specificity of this system is remarkable, and improvements in sensitivity and limit of detection could drive to a major competitivity of its employment in the arsenic biosensors field.

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Chapter [4]: Identification of new heavy metal tolerant *thermophiles*

Extreme environments on our planet are excellent and inexhaustible places to look for microorganisms capable of tolerating stress conditions of temperature, pH, salinity, pressure, and high concentrations of heavy metals.
In the context of this PhD project, the isolation and characterization of new heavy metal tolerant thermophiles is a key point to understand their unique adaptative capabilities and convert them into an attractive source of catalysts for bioremediation and industrial processes.
In this chapter we report the isolation and identification of two new thermophilic microorganisms from the hydrothermally active hot spring of Pisciarelli, near Naples in Italy:

- In Paper I, ribotyping, 16S rRNA sequencing and mass spectrometry analyses lead to the identification of a new strain of *Geobacillus stearothermophilus*, which is tolerant to several heavy metals, and in particular to As(V), as revealed by functional and morphological analyses.
- In Paper II, it is reported the characterization of a new strain of *Alicyclobacillus mali*; the genome analysis highlights the presence of molecular determinants of heavy metal and antibiotic resistance, confirmed by phenotypical analyses consisting on the determination of minimum inhibitory concentrations (MIC) towards these compounds.

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Article

Identification of a New Heavy-Metal-Resistant Strain of *Geobacillus stearothermophilus* Isolated from a Hydrothermally Active Volcanic Area in Southern Italy

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Abstract: Microorganisms thriving in hot springs and hydrothermally active volcanic areas are dynamically involved in heavy-metal biogeochemical cycles; they have developed peculiar resistance systems to cope with such metals which nowadays can be considered among the most permanent and toxic pollutants for humans and the environment. For this reason, their exploitation is functional to unravel mechanisms of toxic-metal detoxification and to address bioremediation of heavy-metal pollution with eco-sustainable approaches. In this work, we isolated a novel strain of the thermophilic bacterium *Geobacillus stearothermophilus* from the solfataric mud pool in Pisciarelli, a well-known hydrothermally active zone of the Campi Flegrei volcano located near Naples in Italy, and characterized it by ribotyping, 16S rRNA sequencing and mass spectrometry analyses. The minimal inhibitory concentration (MIC) toward several heavy-metal ions indicated that the novel *G. stearothermophilus* isolate is particularly resistant to some of them. Functional and morphological analyses suggest that it is endowed with metal resistance systems for arsenic and cadmium detoxification.

Keywords: heavy metals; thermophiles; *Geobacillus stearothermophilus*; minimal inhibitory concentration (MIC); transmission electron microscopy (TEM)

1. Introduction

Heavy metals are among the most persistent and toxic pollutants. Differently from organic xenobiotics, which can be converted into non-harmful substances, they cannot be completely removed from the environment [1]. Anthropogenic sources including mining and various industrial (vehicle emissions, lead–acid batteries, paints) and agricultural activities (fertilizers, aging water supply) lead to their increasing accumulation [2–4]. The prolonged exposure to heavy metals is responsible for several human diseases, as documented by the World Health Organization [5]. For example, arsenic and cadmium have been classified as carcinogenic compounds by the International Agency for Research on Cancer (IARC) in both humans and animals [6,7], while the exposure to lead is responsible for 3% of cerebrovascular disease worldwide [8]. Therefore, reduction of heavy-metal pollution is actually among the greatest challenges of the new century [9–11].

As a consequence of the massive accumulation of toxic metals into the environment, majority of microorganisms have developed genetic resistance mechanisms [12–14] and even specific metabolic

pathways to deal with toxic metals [15–17]. For instance, gram-positive and gram-negative bacteria possess arsenic resistance systems encoded by operons carried either on plasmids or on the chromosome. Genetic determinants are usually the three genes *arsR*, *arsB*, and *arsC* [18,19], among which *arsR* encodes a trans-acting repressor of the ArsR/SmtB family involved in transcriptional regulation [20–22], *arsB* encodes an As(III) efflux transporter (ArsB/Acr3) [23], and *arsC* encodes a cytoplasmic arsenate reductase that converts As(V) to As(III), the latter extruded outside the cell [24–26]. Other organisms benefit of additional proteins that improve the arsenic resistance, such as the arsenite methyltransferases [27]. The arsenic resistance system of some microorganisms also provides Cd(II) tolerance [28–30]. Gram-positive and gram-negative bacteria also possess cadmium resistance systems, which are generally composed by two genes, *cadC* coding for a helix-turn-helix transcriptional regulator that controls the second gene *cadA*, coding for a cadmium-translocating P-type ATPase. The loci of genes involved in cadmium resistance are either on plasmids or on chromosomes [31].

Since many metals, such as arsenic, are naturally present in volcanic and geothermal springs, these niches are commonly colonized by heavy-metal-resistant microorganisms [32,33]; they actively participate in geochemical cycles, solubilizing and precipitating metals thus contributing to transforming the bedrock and remodeling their ecosystems [34]. The interest in the comprehension of the molecular mechanisms underlying heavy-metal resistance in microorganisms thriving in extreme environments is growing fast because of the urgent need to develop effective and eco-sustainable approaches toward heavy-metal pollution [35–38].

In this work, we describe the isolation and characterization of a new thermophilic heavy-metal-resistant microorganism from the solfataric mud pool of Pisciarelli in the Campi Flegrei volcano located near Naples in Italy. The site has extreme environmental conditions in terms of temperature, acidity, and As-rich geochemistry due to an intense hydrothermal activity [39,40].

2. Materials and Methods

2.1. Chemicals

The antibiotics and metal salts used in this work were purchased by Sigma-Aldrich. Antibiotics, identified through the unique number assigned by the Chemical Abstracts Service (CAS), are: Ampicillin (CAS number: 7177-48-2); bleomycin sulfate (CAS number: 9041-93-4); chloramphenicol (CAS number: 56-75-7); kanamycin sulfate (CAS number: 70560-51-9); hygromycin B (CAS number: 31282-04-9); tetracycline (CAS number: 60-54-8). Metal salts used are the following: sodium (meta)arsenite ($NaAsO_2$); sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$); cadmium chloride ($CdCl_2$); cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$); cobalt chloride ($CoCl_3$); chromium oxide (CrO_3); copper nitrate trihydrate [$Cu(NO_3)_2 \cdot 3H_2O$]; mercury chloride, ($HgCl_2$); nickel chloride ($NiCl_2$); sodium orthovanadate dihydrate ($Na_3VO_4 \cdot 2H_2O$).

2.2. Strain Isolation and Molecular Identification

Soil samples were collected in April 2016 at the hydrothermal site of Pisciarelli (Figure 1) that lies on the Solfatara volcano, one of the various eruptive vents generated within the wide volcanic field of the Campi Flegrei since ca. 4100 years [41].

The materials were aseptically sampled from the main mud pool; both pH and temperature were measured contextually by indicator papers and a Fluke digital thermocouple probe, respectively. The water was collected from the bubbling mud pool, while the mud was taken from its marginal water-poorer portion (Figure 1). Temperature and pH values were 94.3 °C and 4.8 in the bubbling mud pool, respectively, while in the marginal water-poorer portion they were 55.3 °C and 6.0, respectively. The local levels of arsenic are in the 10–20 ppm range [39].

Figure 1. Pisciarelli sampling site showing an intense hydrothermal activity and the puddle water (left) and mud (right) collected.

Enrichment cultures were set in 50 mL tubes with 20 mL of Luria-Bertani (LB) medium [42] and inoculated with 1 g of soil sample at 37, 50, and 75 °C for 24 h. Then serial dilutions of these culture samples were streaked on LB-agar plates (1.5%) at the same temperature values for 24 h. Bacterial colonies were found in the mud sample incubated at 50 °C and the isolation of a pure strain was carried out by repeated streak plating on solid medium prepared with the LB-agar incubated at 50 °C.

To identify the new isolate, we used different approaches based on standard procedures such as automated ribotyping on digested chromosomal DNA and fatty acid analysis [43]. Since the taxonomic identification at the species level could not be determined with ribotyping data, a MALDI-TOF MS analysis (i.e., Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry performed with a Time-Of-Flight Mass Spectrometer) was also commissioned to the Leibniz Institute DSMZ (the German Collection of Microorganisms and Cell Cultures, GmbH). Sample preparation and instrumental conditions have been described elsewhere [44]. The identification report was generated by the Biotyper software (version 3.1, Bruker Daltonics GmbH, Bremen, Germany) and the strain was identified as *Geobacillus stearothermophilus* with a score corresponding to high probable strain identification (higher than 2.3).

Moreover, 16S rRNA sequencing was commissioned to Eurofins. The resulting sequencing data (about 1000 nt) were analyzed in the nucleotide database of the U.S. National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The sequence in the sample could be identified as a novel strain of *Geobacillus stearothermophilus* (*G. stearothermophilus* GF16 hereafter). It was also submitted to GenBank and the accession number is MT311361.

Neighbor-Joining Tree Development

In order to obtain information about evolutionary relationships within *G. stearothermophilus* species, the 16S rRNA sequence of the novel *G. stearothermophilus* GF16 isolate was analyzed with BLASTn; sequences with identities from 99% to 97% were aligned using the multiple sequence alignment tool CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Finally, a neighbor-joining tree was constructed using the default option of ClustalW2 (Simple Phylogeny) [45].

2.3. *Geobacillus stearothermophilus* Physiological Analyses

2.3.1. Determination of Optimal Growth Conditions

A frozen (−80 °C) glycerol-stock of *G. stearothermophilus* GF16 was streaked on an LB-agar plate and incubated overnight at 50 °C. Single colonies that appeared on the plate were inoculated into liquid

LB media at different pH values (pH 3, pH 5, pH 7) at 50 and 60 °C under shaking. Growth was only observed at pH 7.0, whereas the optimal growth temperature turned out to be 60 °C. The generation time (G) was calculated with the following formula: $G = t/n$, where t is the time interval and n the number of generations (t is considered to be between 3 and 4 h, in the exponential phase). All the experiments were repeated in triplicate.

2.3.2. Antibiotic Susceptibility

For the determination of minimal inhibitory concentrations (MIC) toward antibiotics, a modified version of a protocol described in the Manual of Antimicrobial Susceptibility Testing was followed [46]. In detail, a frozen (−80 °C) stock of *G. stearothermophilus* GF16 was streaked on an LB-agar plate and incubated at 60 °C overnight. A single colony was inoculated into liquid LB medium and incubated at 60 °C under shaking up to the exponential phase corresponding to Optical Density (OD) at 600 nm of 1.5 (OD_{600nm} were measured in a Varian Cary 50 Scan UV-Visible Spectrophotometer). Then the bacterial culture was diluted up to 0.1 OD_{600nm} in LB medium supplemented with increasing concentrations (from 5 to 50 µg/mL) of antibiotics (ampicillin, kanamycin, chloramphenicol, tetracycline, hygromycin, and bleomycin) and grown at 60 °C for 16 h; for each determination, three independent experiments were carried out in triplicate. Minimum inhibitory concentration (MIC) was determined as the lowest concentration of antibiotics that completely inhibited the growth of the strain as evaluated by OD_{600nm} measurements after incubation for 16 h under optimal growth conditions.

2.3.3. Heavy-Metal Resistance

For the determination of MIC toward heavy-metal ions (As(V), As(III), Cd(II), Co(III), Cr(VI), Cu(II), Hg(II), Ni(II), V(V)), cell cultures were grown and diluted as described above. The heavy metals were added at increasing concentration ranging from 0.1 to 120 mM. The MIC values were determined as described above. The values reported are the average of three independent experiments each one performed in triplicate.

2.3.4. Evaluation of As(V) Biotransformation

The As(V) transformation capacity of *G. stearothermophilus* GF16 to produce As(III) was qualitatively evaluated using a colorimetric assay based on the formation of precipitates upon reaction of $AgNO_3$ with arsenic [47]. A single colony was cultured in LB liquid medium at 60 °C up to 1.0 OD_{600nm}; then an aliquot of the cell suspension was streaked on LB-agar plates containing 50 mM sodium arsenate (Na_2HAsO_4). The LB-agar plates were incubated at 60 °C for 18 h and then flooded with 0.1 M $AgNO_3$. As controls, the following plates were prepared: (1) LB agar supplemented with As(V), without cells; (2) LB agar, without As(V), with streaked cells; (3) LB agar without either cells or As(V). The color of the precipitate on each plate was compared to a color scale, which could be used as reference to distinguish by eye different ratios of As(V)/As(III), thus allowing a qualitative evaluation of As(III) production. The reference color scale was developed by mixing defined ratios of As(V) and As(III) in different tubes. The final concentration of total arsenic was 50 mM for all the samples. All the experiments were repeated in triplicate.

2.3.5. Bioinformatic Analysis

Bioinformatic analyses were performed to evaluate the presence of arsenic and/or cadmium resistance genes in the genomes of the following three sequenced strains of *G. stearothermophilus*: (a) strain n° 10 (Accession BioProject PRJNA252389); (b) strain DSM 458 (Accession BioProject PRJNA327158); (c) strain B5 (Accession Bioproject PRJNA513473). Loci containing sequences coding for putative arsenic resistance proteins were identified on the NCBI database, and the corresponding translated sequences were aligned with the multiple sequence alignment program Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.3.6. Transmission Electron Microscopy (TEM)

To analyze cell morphology, *G. stearothermophilus* GF16 was grown at 60 °C in LB medium (pH 7), and in LB (pH 7) supplemented with As(V) 117 mM or Cd(II) 0.9 mM for 16 h; a control grown in the absence of heavy-metal ions was also harvested at 1.5 OD_{600nm} corresponding to a mid-exponential growth phase. Cells were pelleted by centrifugation, washed twice with phosphate buffered saline (PBS 1%), and fixed as reported by Pinho et al. [48]. Resi-sections were prepared with the ultramicrotome (LKB SuperNova) and serially stained with uranyl acetate and lead citrate. The sections were then studied on a Philips EM 208s Transmission Electron Microscope. Control cells not subjected to metal treatment were compared with heavy-metal-treated samples to check for possible heavy-metal accumulation.

3. Results and Discussion

3.1. Geochemical Characterization of the Sampling Site

Like similar volcanic systems worldwide, the Solfatara volcano hosts an acidic sulfate environment determined by the hot circulation of aggressive sulfurous fluids deriving from mixing between deeply infiltrating meteoric waters and ascending magmatic gases [39–41]. This phenomenon causes intense rock alteration and concentration of certain elements, such as As [39,49–54]. However, differently from the diffuse and fumarolic outgassing characterizing the Solfatara crater, the Pisciarelli site is a water-dominant environment, showing the formation of boiling pools and water springs and the opening of low-energetic geyser-type vents. The site represents the shallowest portion of a widespread geothermal system that develops in the subsurface and converts into brines downward to its deeper roots that are directly supplied by the magmatic outgas. Due to the increased hydrothermal activity since 2006, the site shows maximum temperatures of ca. 110 °C and up to 260 tons/day of CO_2 [55] with an abundance of H_2S and the presence of minor gaseous species such as CH_4, N_2, H_2, and CO.

At the time of sampling, the bubbling mud pool was at pH 4.8 and 94.3 °C and the marginal mud at pH 6.0 and 55.3 °C, while surrounding soils were at temperature up to 98–99 °C and very acidic pH. These values are in the range known for the area, although lower temperatures were also measured in the mud pool (approximately 70 °C). Furthermore, the mineralogical and chemical features of the sampled materials [39] are those usually determined. Indeed, the mud was typically gray in color and essentially enclosed sulfates (i.e., K- and Al- bearing alunite), sulfides (i.e., Fe- plus S-bearing pyrite), and silica-phases; dried water samples crystallized NH_4-bearing sulfates. The mud is enriched in As (10–20 ppm) and Hg (around 40 ppm) compared to the protolith volcanic deposits; contains few wt.% of Fe_2O_3; approximately 60 ppm of V; 10–20 ppm of Pb; <10 ppm of Co, Ni, and Cr; 10–20 ppm of Cu; 1–2 ppm of Tl; and practically lacks Cd being at <0.1 ppm [39]. Based on Valentino and Stanzione [56], Pisciarelli waters are rich in SO_4^{-2} (1400–7000 mg/L) and NH_4 500–1000 mg/L); contain F (0.5–30 mg/L), Al (65–20 mg/L), and B (0.1–0.8 mg/L); lack carbonate species and chlorine; the content of As, Hg, Tl, Pb is approximately 40–2000, 40–250, 2–8, and 5–30 μg/L, respectively. The general enrichment in S, NH_4, As, and Hg is consistent with the volcanic setting and the magmatic/geothermal outgas support. According to Aiuppa et al. [54], arsenate is the As-compost under equilibrium in the water solutions.

3.2. Isolation and Identification of Geobacillus stearothermophilus GF16

Upon incubation of mud samples taken from the marginal water-poorer portion, cell growth was observed in LB medium at pH 7 and 50 °C. Single colonies were isolated by serial dilutions in the same medium, and the isolated strain showed an optimal growth temperature of 60 °C.

In order to identify the microorganism, ribotyping and fatty acid analyses were performed at DSMZ; the results led to the identification of a member of *Geobacillus* genus but did not allow to differentiate unambiguously at species level. Geobacilli were first described by Nazina et al. in 2001 [57]; they are gram-positive, endospore-forming, aerobic or facultative anaerobic thermophiles, growing optimally at temperatures between 50 and 72 °C and exploitable for various biotechnological

Int. J. Environ. Res. Public Health **2020**, *17*, 2678

applications such as for bioremediation and production of thermostable enzymes and biofuels [58,59]. The interest toward microorganisms of the *Geobacillus* genus prompted us to combine two different experimental approaches such as MALDI-TOF MS analysis and 16S rRNA sequencing to try to unambiguously identify the species. Indeed, the classification of the different species within the *Geobacillus* genus is challenging since the sequence similarity of the 16S rRNA can be higher than 97% even among species [60]. On the other hand, MALDI-TOF MS analysis has been proposed as a powerful bioanalytical method to detect profiles of proteins derived from whole bacterial cells to be used for bacteria identification [61]. The combined molecular approaches allowed the identification of a new isolate of *Geobacillus stearothermophilus* that we named *G. stearothermophilus* GF16.

Multiple alignment of 16S rRNA sequence of the novel *G. stearothermophilus* isolate (GF16) with those of other Geobacilli and Bacilli with identities from 99% to 97% was performed to build the phylogenetic tree shown in Figure 2. The results confirmed the difficulty in determining a threshold for defining species within the *Geobacillus* genus and supported the concept that a combination of genotypic and phenotypic characteristics could be not sufficient for describing a new species [60].

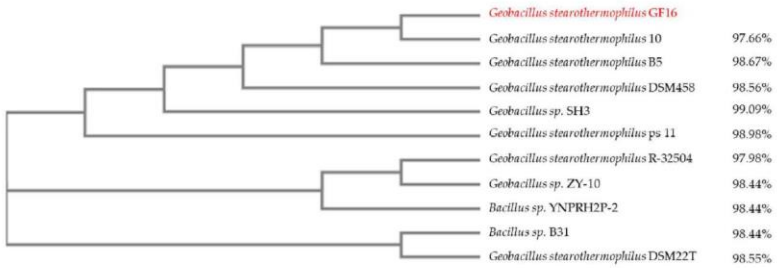

Figure 2. Schematic representation of a phylogenetic tree based on 16S rRNA sequences of different *Geobacillus stearothermophilus* strains. This is a neighbor-joining tree without distance corrections. The new isolate is highlighted in red. The sequence identity (%) to *G. stearothermophilus* GF16 is reported on the right.

3.3. Metal Ion Resistance and Antibiotic Susceptibility in G. stearothermophilus GF16

To evaluate the sensitivity and the tolerance of *G. stearothermophilus* GF16, MICs toward different antibiotics and heavy metals were determined. For this purpose, the microorganism was grown in the presence of different heavy metals (see Table 1) and antibiotics (ampicillin, kanamycin, chloramphenicol, tetracycline, hygromycin, bleomycin). *G. stearothermophilus* GF16 was found to be sensitive to all the tested antibiotics, even at the lowest concentration used; to the best of our knowledge, no antibiotic resistance has been previously reported for other *G. stearothermophilus* isolates, although the genome of *G. stearothermophilus* 10 contains a sequence coding for a putative tetracycline MFS (Major Facilitator Superfamily) efflux protein (locus tag: GT50_RS17520).

Table 1. *G. stearothermophilus* resistance to heavy-metal ions.

Metal Ions	mM	±
As(III)	1.90	0.10
As(V)	117	3.00
Cd(II)	0.90	0.10
Co(II)	2.00	0.50
Co(III)	2.75	0.25
Cr(VI)	0.25	0.01
Cu(II)	4.10	0.10
Hg(II)	0.02	0.00
Ni(II)	1.30	0.10
V(V)	128	2.00

Int. J. Environ. Res. Public Health 2020, 17, 2678

Interestingly, *G. stearothermophilus* showed high tolerance to As(V) and V(V), as reported in Table 1. Similar MIC values were also found in other Geobacilli such as *G. stearothermophilus* AGH-02 [62], *G. stearothermophilus* ASR4 [63], or *Geobacillus kaustophilus* [64]. The high resistance to both vanadate and arsenate ions was not surprising considering the similarity in their structures; in addition, the structural similarity of both ions with the phosphate ions suggested that V(V) and As(V) could be taken up by cells through phosphate transport systems [65].

As for other aerobic microorganisms [66], arsenic resistance within the *Geobacillus* genus relies on the ability to oxidize arsenite, or to reduce arsenate and extrude the arsenite. In particular, As(III) resistance depend on membrane or periplasmatic oxidase activities [67], while resistance to As(V) mainly involves intracellular reductase activities [68] and membrane transporters for As(III) efflux [69]. Since we measured very low tolerance to As(III) (Table 1) in comparison to the values reported in the literature (1.9 mM versus 10–30 mM) [62–64], it can be hypothesized either that our isolate lacks arsenite oxidase activity or that the high sensitivity to As(III) is due to the absence of active transport systems for As(III) efflux. For example, the legume symbiont *Sinorhizobium meliloti* was very tolerant to As(V) but very sensitive to As(III) since it was deficient of As(III) transporter systems [65,70].

The new isolate was also found to be Cd(II) tolerant, and in this case the MIC value determined was similar to that measured in other Geobacilli (ranging from 0.4 to 3.2 mM) [71]. For the majority of these microorganisms Cd(II) resistance was ascribed to biosorption, i.e., a phenomenon of metal binding to the microbial cell wall, which does not involve energy consumption [72,73]. Interestingly, the Pisciarelli site is enriched in arsenic and vanadium but lacks cadmium (see Section 3.1). Therefore, the presence of genetic determinants for Cd(II) tolerance cannot be traced back to the selective pressure exerted by the environment.

Figure 3 shows the effect of As(V) on *G. stearothermophilus* GF16 growth: the generation time shifted from 30 min for cells grown in the absence of As(V) to 60 and 125 min for those grown in the presence of As(V) 25 and 50 mM, respectively.

As we only observed a high As(V) resistance, we sought to evaluate whether *G. stearothermophilus* GF16 had any As(V) reductase activity; for this purpose, an $AgNO_3$ colorimetric method [47,74–76] was employed on cells grown on LB-agar supplemented with As(V), using as controls plates of: (1) LB-agar with As(V) and no cells; (2) LB-agar without As(V) and with grown cells; (3) LB-agar without cells and As(V) (Figure 4). The method is based on the formation of colored precipitates upon reaction of $AgNO_3$ with arsenic; in particular, the addition of $AgNO_3$ to the grown cells produces a brown precipitate (Ag_3AsO_4) if $AgNO_3$ reacts with As(V) and a bright yellow precipitate (Ag_3AsO_3) if $AgNO_3$ reacts with As(III) [47,74–76]. Therefore, the addition of As(V) to the growth medium implies that As(III) can be revealed only if it is produced inside the cell and extruded afterward. Moreover, as can be seen from the reference color scale in Figure 4E, the solution is clearly yellow only when As(III) is more than 50% of the total arsenic. Figure 4A shows a brown precipitate, indicating that As(V) was the predominant chemical species outside the cells. The negligible amount of extracellular As(III) detected within this experiment, suggested either that *G. stearothermophilus* GF16 had low As(V) reductase activity or could not efficiently extrude As(III). This latter hypothesis might be consistent with the lack or low activity of As(III) efflux systems. However, to confirm these hypotheses, more sensitive experimental approaches such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS), able to detect lower amounts of As(III), are required.

3.4. Bionformatic Analyses

To the best of our knowledge, the genomes of only three strains of *G. stearothermophilus* GF16 have been fully sequenced (Table 2): (1) strain "10" isolated from the Yellowstone hot spring (USA); (2) strain "DSM458" isolated from a sugar beet factory in Austria [77]; and (3) strain "B5" isolated from a rice stack in China. As shown in the phylogenetic tree, these strains are evolutionarily very closely related to the GF16 isolate (Figure 2).

Int. J. Environ. Res. Public Health **2020**, *17*, 2678

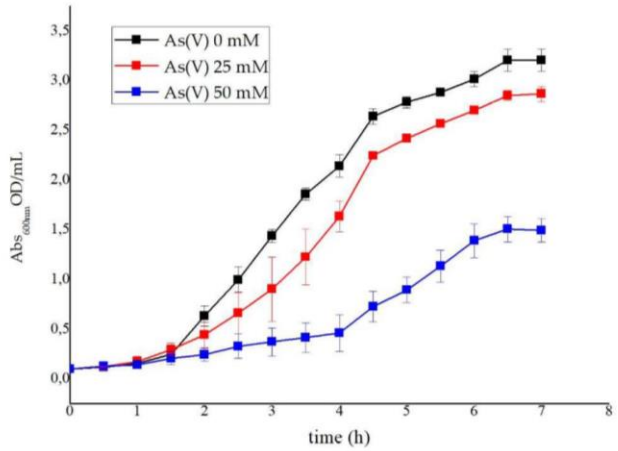

Figure 3. *G. stearothermophilus* GF16 grown in the presence and absence of As(V).

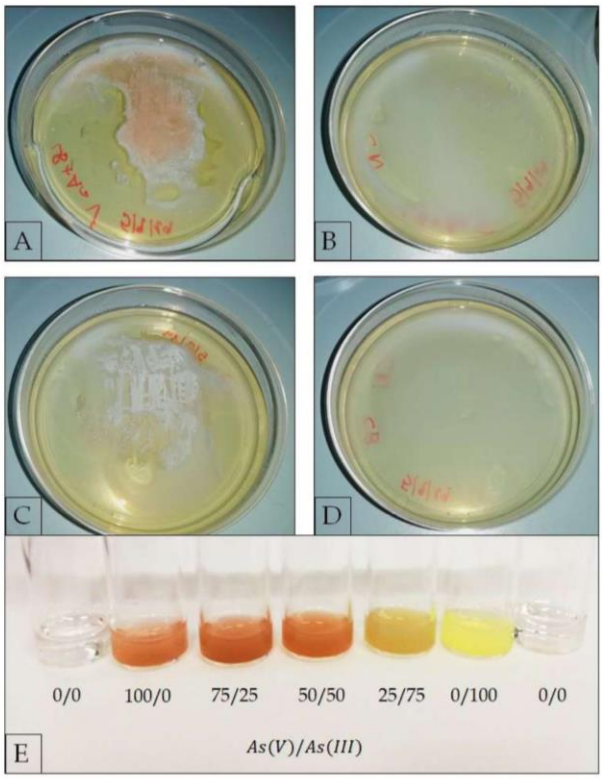

Figure 4. Silver nitrate test on: (**A**) *G. stearothermophilus* GF16 grown on LB-agar plate supplemented with 50 mM As(V); (**B**) LB-agar plate supplemented with 50 mM As(V) (control 1); (**C**) *G. stearothermophilus* grown on LB-agar plate (control 2); (**D**) LB-agar plate (control 3). (**E**) Determination of precipitate color as function of As(V)/As(III) ratio (%). The concentration of total arsenic in solution in each sample is 50 mM (i.e., for the ratio 50/50 there are in solution As(V) 25 mM and As(III) 25 mM).

Table 2. List of *G. stearothermophilus* strains with sequenced genomes, as reported in the National Center for Biotechnology Information (NCBI) genome databank.

Organism	Strain	Origin	Genome Size (Mb)	CG%	Gene	Protein	BioProject
Geobacillus stearothermophilus	10	Yellowstone thermal spring	3.67	52.61	3645	3312	PRJNA252389
Geobacillus stearothermophilus	DSM458	Austria sugar beet factory	3.46	52.10	3683	3165	PRJNA327158
Geobacillus stearothermophilus	B5	China rice stack	3.39	52.50	3426	3045	PRJNA513473

In order to verify whether different strains of *G. stearothermophilus* had arsenic and cadmium resistance systems and to understand whether such systems were conserved among the species, a comparative genomic analysis was carried out on the sequences of three *G. stearothermophilus* genomes available at NCBI (Table 2). The study revealed differences in the abundance and type of putative arsenic and cadmium resistance genes in the genomes analyzed (Table 3). In particular, all of them contained one conserved copy of *cadC* and *cadA*: the alignment of the corresponding proteins from the three different strains showed a high degree of identity (92%). This result could explain the tolerance of the new isolate toward Cd(II) despite the absence of this metal within its specific environment (see Section 3.1). Regarding arsenic resistance systems, a copy of ArsB/Acr3 arsenite efflux transporters was found in each genome, whereas at least a simple *ars* system encoding the arsenate reductase (*arsC*) in tandem with an ArsR/SmtB transcriptional regulator was found only in the genomes of *G. stearothermophilus* 10 and B5 strains (86% of identity of both proteins). On the other hand, the strain DSM458 encoded a unique arsenate reductase; moreover, sequences coding for putative arsenite oxidases were not observed in any of the genomes analyzed [78,79]. To the best of our knowledge there are no reports of functional studies on metal resistance systems in these three strains, therefore we conclude that additional investigation is required to shed light on the occurrence of common metal resistance mechanisms in *G. stearothermophilus* isolates. However, the in silico analysis of the genomes showed that the number and type of genes coding for elements involved in arsenic resistance is variable within the same species and depends on the specific evolutionary adaptation of that particular strain [80].

Table 3. List of putative genes for As and Cd(II) resistance in *G. stearothermophilus* strains.

	***Geobacillus stearothermophilus* 10 (PRJNA252389)**	***Geobacillus stearothermophilus* DSM458 (PRJNA327158)**	***Geobacillus stearothermophilus* B5 (PRJNA513473)**
Putative Proteins	***Locus***	***Locus***	***Locus***
ArsR	GT50_RS07590		EPB69_RS07030 EPB69_RS15665 EPB69_RS15730
ArsB	GT50_RS07510	GS458_RS16835	EPB69_RS15660
ArsC	GT50_RS07505 GT50_RS06280	GS458_RS16830 GS458_RS15800	EPB69_RS15655
CadA	GT50_RS12470	GS458_RS03700	EPB69_RS03440
CadC	GT50_RS12465	GS458_RS03695	EPB69_RS03435

3.5. Analysis of Cellular Morphology

In order to better define *G. stearothermophilus* GF16 morphology, we resolved to analyze cells through TEM. As shown in Figure 5, cells have a typical bacillar rod shape when they are actively growing.

Moreover, with the aim of verifying whether As(V) and Cd(II) had any effect on cell morphology, TEM images were also acquired on samples of *G. stearothermophilus* GF16 grown for 16 h in the presence of As(V) and Cd(II) at concentrations corresponding to the MIC values, and they were compared to images of control cells not subjected to any treatment with heavy metal (Figure 6). The sections obtained revealed the structure of the cell more clearly in the control cells (Figure 6A) than in those treated with heavy metals. However, the presence of several cells in division suggested that both As(V) and Cd(II) did not cause significant changes in the cellular structure and cell viability (of Figure 6B vs.

Int. J. Environ. Res. Public Health **2020**, *17*, 2678

Figure 6C). Nevertheless, it appeared that the cell wall of *G. stearothermophilus* GF16 was influenced by handling both As and Cd. In particular, the cell wall of *G. stearothermophilus* GF16 treated with As(V) (Figure 6B) exhibited abundance of ridges and grooves that can be related to a reduction in cell permeability. Interestingly, Cd(II)-treated cells (Figure 6C) appeared darker; this phenomenon could be ascribed to the ability of *G. stearothermophilus* to adsorb Cd(II), as also reported by Hetzer et al. [71].

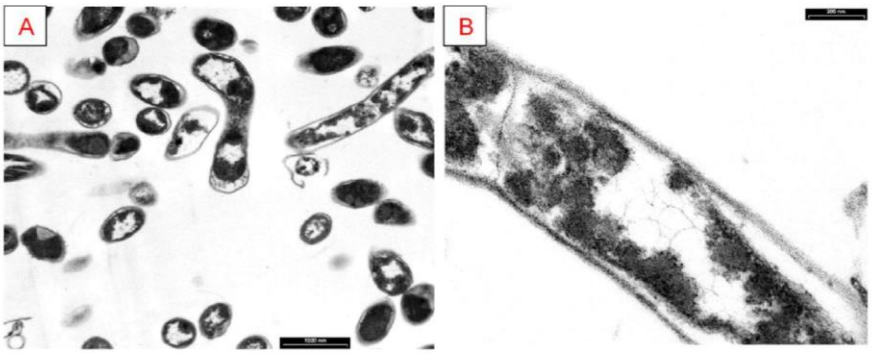

Figure 5. TEM images of *G. stearothermophilus* GF16 in exponential phase at different scales (black bars): (**A**) 1000 nm; (**B**) 200 nm.

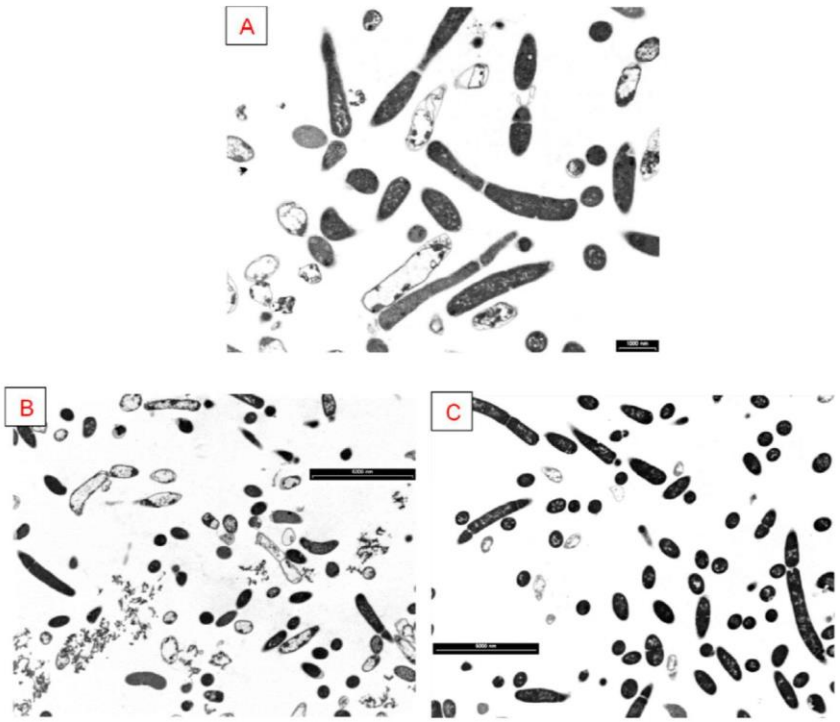

Figure 6. TEM images of *G. stearothermophilus* GF16: after overnight growth (**A**), in the presence of As(V) (**B**) and Cd(II) (**C**) at their respective MIC values. Scale: 1000 nm in **A**, elsewhere 6000 nm.

In conclusion, electron microscopy analyses highlighted that the cell shape/structure of *G. stearothermophilus* GF16 changes in presence of As(V) and Cd(II), thus suppling a morphological explanation for the tolerance of the new isolate toward these metal ions.

4. Conclusions

With the aim of characterizing new thermophilic heavy-metal-resistant microorganisms, soil sampling was performed in a hydrothermal volcanic area near Naples in Italy, known as Pisciarelli. This is an acidic sulfate area located close to the Solfatara crater famous for an intense endogenous diffuse and fumarolic water-dominant outgassing activity; the chemical composition of mud and water samples revealed that the main metal is iron, but arsenate is an additional significant component. Since geothermal sites are very interesting sources of thermophilic organisms and Pisciarelli is an arsenic-rich area, we hypothesized that novel thermophiles could be found able to detoxify this metal or use it for energy-yielding reactions. We succeeded in isolating a microorganism with an optimal growth temperature of 60 °C and an optimal pH 7, from a water-poor mud. Subsequent molecular identification revealed homology to the species *G. stearothermophilus*. Our laboratory culturing experiments demonstrated the ability of *G. stearothermophilus* GF16 to grow in the presence of arsenate in a range of concentrations comparable to those of bacteria classified as arsenic resistant and in agreement with the natural environmental setting composition as well. This study highlights the adaptation capabilities of the new isolate of *G. stearothermophilus* and its tolerance to extreme environmental conditions and points out to further molecular and physiological investigations to clarify its role in the biogeochemical cycle of arsenic as well as its potential for the management of heavy-metal environmental contaminations.

Author Contributions: Data curation, R.P.; investigation, R.P., G.G., M.P., A.M., S.B., and G.F.; methodology, R.P., G.G., M.P., A.M., and G.F.; supervision, D.L., P.C., M.P., S.B., and G.F.; writing—review and editing, M.P. and G.F. All authors have read and agree to the published version of the manuscript.

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Genomic Insight of *Alicyclobacillus mali* FL18 Isolated From an Arsenic-Rich Hot Spring

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Extreme environments are excellent places to find microorganisms capable of tolerating extreme temperature, pH, salinity pressure, and elevated concentration of heavy metals and other toxic compounds. In the last decades, extremophilic microorganisms have been extensively studied since they can be applied in several fields of biotechnology along with their enzymes. In this context, the characterization of heavy metal resistance determinants in thermophilic microorganisms is the starting point for the development of new biosystems and bioprocesses for environmental monitoring and remediation. This work focuses on the isolation and the genomic exploration of a new arsenic-tolerant microorganism, classified as *Alicyclobacillus mali* FL18. The bacterium was isolated from a hot mud pool of the solfataric terrains in Pisciarelli, a well-known hydrothermally active zone of the Campi Flegrei volcano near Naples in Italy. *A. mali* FL18 showed a good tolerance to arsenite (MIC value of 41 mM), as well as to other metals such as nickel (MIC 30 mM), cobalt, and mercury (MIC 3 mM and 17 μM, respectively). Signatures of arsenic resistance genes (one arsenate reductase, one arsenite methyltransferase, and several arsenite exporters) were found interspersed in the genome as well as several multidrug resistance efflux transporters that could be involved in the export of drugs and heavy metal ions. Moreover, the strain showed a high resistance to bacitracin and ciprofloxacin, suggesting that the extreme environment has positively selected multiple resistances to different toxic compounds. This work provides, for the first time, insights into the heavy metal tolerance and antibiotic susceptibility of an *Alicyclobacillus* strain and highlights its putative molecular determinants.

Keywords: geothermal environment, thermophilic microorganism, toxic metals, genomic sequencing and annotation, arsenic resistance system, bioremediation

Abbreviations: As(V), arsenate; As(III), arsenite; Sb(III), antimony; Cd(II), cadmium; Cu(II), copper; Co(II), cobalt; Ni(II), nickel; Hg(II), mercury; MIC, Minimal Inhibitory Concentration; IARC, International Agency of Research on Cancer; UPP, undecaprenyl-pyrophosphate.

INTRODUCTION

Arsenic is a widespread toxic metalloid component of the Earth crust and present in many geothermal environments (Nagy et al., 2014). It is also released into the environment by the consumption of arsenic-containing products such as insecticides, pesticides, and chemotherapeutic drugs (Bhowmick et al., 2018). Arsenic exists in two oxidation states, As(III) and As(V), and in inorganic and/or organo-metalloid forms (Qin et al., 2006). Due to its redox-active nature, arsenic is highly toxic as it may interfere in several biochemical reactions, by binding thiol groups of proteins or by substituting phosphate in oxidative phosphorylation (Nagy et al., 2014). Since arsenic causes numerous human diseases, it has been ranked by the World Health Organization among the top 10 chemicals that threaten public health and a class-I human carcinogen on the list of the IARC (International Agency for Research on Cancer, 2002; Mandal and Suzuki, 2002).

Geothermal environments are considered extreme habitats, not compatible with human life, because they combine different hostile conditions such as high temperature, salinity, acidity, and relevant metal concentrations; indeed, in acidic environments, the metal solubility is higher than in neutrophilic environments (Norambuena, 2020) and the levels of total arsenic can be up to 50 mg/l (Ballantyne and Moore, 1988). Such peculiar niches also represent exceptional reservoirs of poly-extremophiles, i.e., microbes adapted to live in the presence of more than one extreme condition (Saxena et al., 2017; Gallo et al., 2018; Schmid et al., 2020). Extremophilic microorganisms have attracted scientists for many reasons: they teach us about the origin and the limits of life (Bartolucci et al., 2013; Fusco et al., 2020); the use of their proteins/enzymes has boosted significant industrial and technological advances (Aulitto et al., 2018, 2019b; Fiorentino et al., 2020; Nayak et al., 2020); the elucidation of their molecular adaptations to survive in harsh conditions has revealed unique metabolic pathways and highlighted their potential as robust chassis for metabolic engineering (Fiorentino et al., 2003; Qin et al., 2006; Pothier et al., 2018). Application of extremophiles can further improve sustainability of biotechnological processes that can run more efficiently under harsh conditions (Krüger et al., 2018).

The exploration of the microbial diversity, the assessment of arsenic tolerance in volcanic water systems, and the microbial contribution to arsenic speciation are emerging fascinating fields covering culture-dependent and culture-independent approaches (Langner et al., 2001; Cuebas et al., 2011; Hug et al., 2014); in this regard, -omics techniques, such as metagenomics, genome sequencing, transcriptome and proteome analyses, and quantitative arsenic speciation data boost information on the complex relationship between chemical speciation and microbial metabolism and on the elucidation of the molecular mechanisms of arsenic tolerance in extreme environments (Langner et al., 2001; Wilkin et al., 2003; Aiuppa et al., 2006; Farnfield et al., 2012).

Such knowledge has set the basis to exploit microbial arsenic bioprocesses for the development of eco-sustainable approaches to deal with heavy metal pollution as well as to set up protein-based or whole-cell biosensors for the detection of heavy metals (Gomes et al., 2016; Politi et al., 2016; Krüger et al., 2018; Norambuena, 2020; Puopolo et al., 2021).

Microbes employ a variety of strategies to detoxify arsenic that include biochemical transformation (e.g., redox processes or methylation), extracellular precipitation, intracellular sequestration, and active extrusion from the cells (Páez-Espino et al., 2009).

The arsenic resistance genes are usually organized in *ars* operons, which include either three (*arsRBC*) or five (*arsRDABC*) genes: *arsR*, coding for As(III)-responsive transcriptional repressor controlling expression of *ars* genes; *arsC* encoding an arsenate reductase, which confers resistance to arsenate by converting arsenate into arsenite (Messens et al., 2002; Del Giudice et al., 2013); and *arsB* encoding an As(III) efflux protein. Acr3 is an unrelated As(III) antiporter (around 20–40% sequence similarity) that can be found as an alternative to ArsB (Yang et al., 2012), *arsA* encodes an As(III) stimulated ATPAse (Carlin et al., 1995), and *arsD* is a metallochaperone that favors arsenite transfer to ArsAB (Lin et al., 2006). The *arsRBC* operon is present in several microbes, among them *Staphylococcus aureus* plasmid pI258 (Ji and Silver, 1992), whereas *arsRDABC* has been characterized in *Escherichia coli* plasmid R773 and in other microorganisms (Saltikov and Olson, 2002). Many microbes possess an additional gene, *arsM*, coding for an arsenite methyltransferase that catalyzes the conversion of inorganic arsenic into mono-, di-, and tri-methylated products (Huang et al., 2018; Mestrot et al., 2020).

Among bacterial and archaeal extremophiles, enzymes for arsenic redox transformations and resistance systems have been characterized; for example, *Thermus* species use arsenate as the final acceptor of oxidative phosphorylation (Gihring and Banfield, 2001) and *T. thermophilus* HB27 has an arsenic resistance system composed of an arsenate reductase, an ArsR/SmtB transcriptional regulator, and an As(III) efflux transporter (Antonucci et al., 2017; Gallo et al., 2019). The extremely resistant archaeon *Ferroplasma acidarmanus* co-transcribes and expresses *arsB* and *arsR* in response to As(III), by compensating the lack of *arsC* in the genome (Gihring et al., 2003); other phylogenetically distinct archaea including *Pyrobaculum calidifontis*, *Sulfolobus tokodaii*, and *Aeropyrum pernix* are As(III)-oxidizing microbes that use a membrane arsenite oxidase encoded by widespread *aio* clusters (Mikael Sehlin and Börje Lindström, 1992; Shi et al., 2020).

A considerable number of microbial genomes has highlighted even greater complexity of the arsenic resistance determinants; for example, resistance genes can be often found scattered in the genome, duplicated or present in multiple copies; it has been suggested that redundancy of gene configurations in *ars* gene clusters has arisen from horizontal gene transfer and is responsible for the increased arsenic tolerance (Ordóñez et al., 2005; Li and Krumholz, 2007; Páez-Espino et al., 2015). In extremophilic microorganisms, genomic islands encoding for multiple resistances would have been acquired by the same microbe, conferring it capabilities to face multiple metals (Jackson et al., 2005; Norambuena, 2020).

The present study aimed to deepen knowledge on the genetic determinants of heavy metal tolerance, particularly arsenic, in microbes thriving in extreme environments; in this context, we chose as isolation site Pisciarelli, a hot spring located in the volcanic area of Campi Flegrei in Italy. It displays an impressive and powerful hydrothermal activity with a puzzling of various hot and acidic environments (Baquero et al., 2020) induced by fumaroles at temperature up to ∼110°C, vigorous boiling pools and diffuse soil degassing. From year 2006, these conditions have been continuously changing and increasing, due to the effect of the endogenous engine and the meteoric agents (Valentino and Stanzione, 2003; Chiodini et al., 2010; Piochi et al., 2015, 2019; Cardellini et al., 2017).

Therefore, we describe herein the isolation of a novel thermoacidophilic bacterium, *Alicyclobacillus mali* FL18, from a hot mud pool at Pisciarelli and its genomic and physiological characterization.

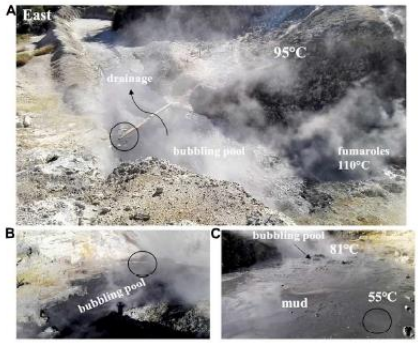

FIGURE 1 | The Pisciarelli site, the sample mud pool, and the sample area (white circle). **(A)** The hydrothermal setting with the fumaroles, the main boiling pool, the outflow water drainage zone, and the surrounding diffuse degassing area. **(B)** Southern view of the bubbling pool with the boiling points. **(C)** The sampled muddy area at the periphery of the mud pool.

MATERIALS AND METHODS

Chemicals

The metal salts used in this work were purchased by Sigma-Aldrich and they are as follows: Sodium (meta) arsenite ($NaAsO_2$), Sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$), Cadmium chloride ($CdCl_2$), Cobalt chloride ($CoCl_3$), Copper chloride ($CuCl_2$), Mercury chloride ($HgCl_2$), and Nickel chloride ($NiCl_2$). The antibiotics used were also purchased by Sigma-Aldrich and are ampicillin (CAS Number: 7177-48-2), bacitracin (CAS Number: 1405-87-4), chloramphenicol (CAS Number: 56-75-7), ciprofloxacin (CAS Number: 85721-33-1), erythromycin (CAS Number: 114-07-8), kanamycin sulfate (CAS Number: 70560-51-9), streptomycin (CAS Number: 3810-74-0), tetracycline (CAS Number: 60-54-8), and vancomycin (CAS Number: 1404-93-9).

Study of the Area and Sampling

The samples were collected in September 2018 at Pisciarelli (**Figure 1A**), an active portion of the wider volcanic field of Campi Flegrei close to Naples in Italy. The area located on the northeastern slope of the Solfatara cone degasses sulfurous water vapors and over 300 g/m^2 per day of CO_2 (Chiodini et al., 2010; Piochi et al., 2015; Cardellini et al., 2017). This hot spring is characterized by the presence of a main bubbling mud pool with temperatures up to 80–85°C and marginal water-poorer portions with lower temperature (Piochi et al., 2019). The levels of arsenic are included in the 10–15 ppm range in the mud and in the 39–2,000 μg/L range in waters reaching up to 6,000 μg/L in nearby zones (Valentino and Stanzione, 2003). The samples were aseptically collected close to the marginal water-poorer portion (**Figures 1B,C**) where the temperature and the pH were ∼55°C and 5.0, respectively.

Cultivation and Isolation of Growing Bacteria

To select thermophilic and acidophilic bacteria, the collected samples were enriched in 20 ml of modified Luria-Bertani (LB) (adjusted to pH 5 through addition of HCl) and incubated for 24 h at 55°C with a shaking rate of 180 rpm, in a MaxQTM 4000 Benchtop Orbital Shaker (Thermo Scientific). The enriched sample was plated on LB agar pH 5.0 at 55°C and grown for 48 h. Single colonies were isolated through repeated streak-plating and then the isolate was identified through 16S rRNA gene sequencing followed by data analysis through the EMBL database[1]. The sequence could be identified as belonging to a member of the genus *Alicyclobacillus*.

Genomic DNA Isolation and Sequencing

Genomic DNA extraction from *Alicyclobacillus* spp. was performed using LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris–HCl, pH 7.4, and 0.2% SDS) and phenol extraction as already described (Aulitto et al., 2019a). Final yield and quality of DNA were determined spectrophotometrically using a Qubit Fluorometer (Invitrogen Co.). One hundred nanograms of genomic DNA was then used to prepare indexed libraries with Nextera DNA Flex Library Prep (Illumina) according to the manufacturer's instructions. The library was quantified using Qubit fluorometer (Invitrogen Co.) and then sequenced on NextSeq550 platform (Illumina, San Diego, CA, United States) in a 2 × 75 paired-end format. Whole-genome sequencing was performed at Genomix4life s.r.l. (Salerno, Italy).

Genome Assembly and Annotation

A total of 2,309,234 reads (average length, 75 bp) were assessed through FastQC v 0.11.5 (Brown et al., 2017). Different assemblers SPAdes v 3.13.0 (Bankevich et al., 2012), IDBA-UD v 1.1.3 (Peng et al., 2012), and MEGAHIT v 1.2.9 (Li et al., 2015) were used and the results compared using QUAST v 4.4 (Mikheenko et al., 2016). Furthermore, the assembly was

[1]https://www.ebi.ac.uk/ena

evaluated for completeness and contamination by means of CheckM v 1.0.18 (Parks et al., 2015). For comparative genomics and gene analysis, a Rapid Annotations using Subsystems Technology (RAST) v 0.1.1 was used to perform the annotation (Aziz et al., 2008). This analysis was used to identify (i) arsenic resistance genes, (ii) heavy metal resistance genes, and (iii) antibiotic resistance genes, by performing BLASTp and HMM-based procedures included in RAST system. EggNOG (Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups) database was employed to assign a functional annotation of the identified orthologous groups and to hamper the interpretation of subsequent results (Huerta-Cepas et al., 2019). The orthologous clusters were visualized using the online service OrthoVenn2[2] (Xu et al., 2019).

Comparative Genomic Analysis

To construct the phylogenetic tree, different sequences of *Alicyclobacillus* species available at National Center for Biotechnology Information (NCBI) genome sequence repository[3] were compared using SpeciesTreeBuilder v.0.1.

The average nucleotide identity (ANI) was determined using the online ANI calculator based on an improved OrthoANIu algorithm[4]. *In silico* DNA–DNA hybridization (DDH) values were estimated using the Genome-to-Genome Distance Calculator (GGDC)[5]. ANI values of 95–96% and DDH value of 70% were used as a boundary for species delineation. A graphical genome view was generated employing CGView software (Grant and Stothard, 2008).

A. mali FL18 Growth Conditions

In order to identify the optimal growth conditions of *A. mali* FL18, growth curves were followed in three different media: Luria Bertani medium (LB) pH 4.0, Yeast Starch Glucose medium (YSG) pH 4.0, and *Bacillus acidoterrestris* thermophilic medium (BAT, HiMedia Biolabs), pH 4.0 (Anjos et al., 2014). A frozen (−80°C) glycerol-stock of *A. mali* FL18 was incubated in 5 ml of each medium for 16 h at 55°C and then diluted at 0.1 $OD_{600\ nm}$ in fresh medium, and the growth measured shaking for 16 h at 55°C in a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, United States). Each experiment was performed in technical and biological triplicates.

Heavy Metal Ion Tolerance

For the determination of Minimal Inhibitory Concentration (MIC) toward the heavy metal ions [As(V), As(III), Cd(II), Co(III), Cu(II), Hg(II), Ni(II)], exponentially growing cells were diluted to 0.1 $OD_{600\ nm}$ in 600 µl of BAT medium (pH 4.0) containing metal ion at concentrations ranging from 0.5 µM to 45 mM. The cultures were incubated at 55°C for 16 h and MIC values were determined as the lowest concentration of metals that completely inhibited the growth of *A. mali* FL18 adopting the procedure already described (Antonucci et al., 2017, 2018). The reported values are the average of three biological replicates.

Determination of Arsenic Tolerance

To evaluate the effect of arsenate and arsenite on the bacterial growth, an overnight culture was diluted at 0.1 $OD_{600\ nm}$ in fresh BAT medium and grown in the presence of 20 mM As(III) and 6 mM As(V). The growth curves were recorded measuring the $OD_{600\ nm}$ for 16 h at 55°C under shaking in a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, United States). The generation time (G) was calculated as: $G = t/n$, where t is the time interval and n the number of generations (measured between 2 h and 3 h, in the exponential phase). All the experiments were repeated in triplicate.

Antibiotic Susceptibility

MIC toward different antibiotics was determined using a procedure previously described (Puopolo et al., 2020). In detail, the bacterial culture was diluted up to 0.1 $OD_{600\ nm}$ in BAT medium, pH 4.0, supplemented with concentrations ranging from 0.5 to 1,000 µg/ml of ampicillin, bacitracin, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, streptomycin, tetracycline, and vancomycin, and grown at 55°C for 16 h. MIC was evaluated by measuring $OD_{600\ nm}$ after incubation for 16 h in the presence of different concentrations of antibiotics. The reported values are the average of three biological replicates.

Arsenic Biotransformation by *A. mali* FL18

A colorimetric assay based on the formation of precipitates upon reaction of $AgNO_3$ with arsenic was set up to address biotransformation of arsenate into arsenite, and *vice versa*, by *A. mali* FL18 (Simeonova et al., 2004). A single colony was inoculated in BAT medium, pH 4.0, at 55°C for 6 h, and then it was diluted to 0.1 $OD_{600\ nm}$ in 1 ml of BAT medium containing 5 mM Na_2HAsO_4 and 20 mM $NaAsO_2$; an arsenic-free control culture (CN) was also grown. The cultures were incubated at 55°C for 16 h, and cells were harvested by centrifugation at 6,000 rpm for 10 min (Eppendorf Centrifuge 5804 R); the supernatants and pellets were flooded with 100 µl of 0.1 M $AgNO_3$. The color of the precipitate on each sample was compared to two reference color scales each obtained by mixing defined ratios of As(V) and As(III) (100:1, 75:25, 50:50, 25:75, and 1:100) at final concentrations of total arsenic of 5 mM or 20 mM. This analysis gives a qualitative indication of arsenic transformation. All the experiments were repeated in triplicate.

RESULTS AND DISCUSSION

Site Description, Sampling, and Identification of a New Strain of *Alicyclobacillus*

This study focuses on the isolation and identification of a heavy metal tolerant bacterium from a well-known solfataric

[2]https://orthovenn2.bioinfotoolkits.net/
[3]https://www.ncbi.nlm.nih.gov/genome
[4]https://www.ezbiocloud.net/tools/ani
[5]http://ggdc.dsmz.de/ggdc.php#

environment, located in the volcanic area of Campi Flegrei close to Naples, Italy. Hot springs are unique niches of biodiversity, and microbes living under intense selective pressure are found to be part of consortia sharing temperature, pH, *in situ* chemistry, and biogeography (Portillo et al., 2009). In these communities, microorganisms have evolved strategies to thrive in harsh conditions by converting chemicals and organic matter into cellular energy (Aulitto et al., 2020). In the biogeochemical cycle of arsenic, the microbiome plays a key role contributing to metal mobilization as well as to shape the structure of the ecosystem in which it thrives (Langner et al., 2001; Donahoe-Christiansen et al., 2004).

Pisciarelli was selected as the ideal isolation site because, as known for other volcanic areas, it is characterized by aggressive sulfurous effluxes and significant concentration of certain elements such as arsenic and mercury over 50 ppm (Valentino and Stanzione, 2003; Piochi et al., 2019). It has been shown that sampling in acid-sulfidic geothermal systems can give insight into the effects of microbial communities on arsenic and sulfur biogeochemical cycles and increase the knowledge on the relationship between arsenic resistance and sulfur cycling (Hug et al., 2014).

Table 1 summarizes main physical–chemical features of the Pisciarelli site. In particular, regarding sulfur forms, H_2S is the main gas species, whereas in the water and mud/soil, the stable form is SO_4^{2-}. Pyrite (FeS_2) and native sulfur (S_8) occur within the mineral association of mud and soil; total S is between ca. 11 and 30% in mud, and SO_4^{2-} is between ca. 1,300 and 7,100 mg/L in water (**Table 1**). Regarding arsenic forms, As(V) oxy-anions ($HAsO_4^{2-}$, AsO_4^{3-}) are predominant, in agreement with the highly oxidant conditions and the geothermal outgas (Aiuppa et al., 2006). Moreover, dimeric As-S complexes ($H_2As_2S_4$) are the major As aqueous species at equilibrium under sulfur-rich conditions.

The occurrence of heavy metals is also reflected by the presence of mineral precipitates, i.e., realgar (As_4S_4) and pyrite

TABLE 1 | Physico-chemical conditions at the Pisciarelli site.

		Solid	Water	Fumarole
Temperature (°C)	94.3		83–96[3]	116
pH	4.8		1.4–4.9[3]	n.d.
TDS			2060–9100[3]	
Minerals[1]	–	Alunite, sanidine, pyrite, sulfur, hydroBiotite/illite, amorphous	Illite/montmorillonite, ammonium K sulfate	–
Main sulfur state	SO_4^{2-}	SO_4^{2-}, S^0, sulfides (mostly S^{-1})	SO_4^{2-}	H_2S
H_2S flux	0.091 tons/day[2]	–	–	0.849 tons/day[2,3]
SO_2 flux	n.d.	–	–	0.0014 tons/day[2]
CO_2 flux	48.3 tons/day[2]	–	–	231–300 tons/day[2,3]
H_2 flux	0.0033 tons/day[2]	–	–	0.0227 tons/day[2]
H_2O content	–	–	–	809,000–870,000 μmol/mol[4]
CO_2 content	–	–	–	115,000–188,000 μmol/mol[4]
H_2S content	–	–	–	517–696 μmol/mol[4]
H_2 content	–	–	–	137–294 μmol/mol[4]
CH_4 content	–	–	–	9.1–19 μmol/mol[4]
Al (as Al_2O_3 in solid)	–	10–11.85 wt%[5]	19–66 mg/L[6]	–
Na (as Na_2O in solid)	–	0.24–0.42 wt%[5]	7.7–122 mg/L[6]	–
Fe (as Fe_2O_3 in solid)		2.3–3.1 wt%[5]	34–161 mg/L[6]	
Cl	–	n.a.	6–83 mg/L[6]	–
NH_4^+	–	n.a.	501–1026 mg/L[6]	–
HCO_3^{2-}	–	–	n.d.	–
SO_4^{2-} (as S in solid)	–	10.62–31.12 wt%[5]	1319–7043 mg/L[6]	–
F	–	n.a.	0.1–30 mg/L[6]	n.d.
C	–	0.14–0.21 wt%[5]	–	–
As	–	9.5–12.9 ppm[5]	39–1880 μg/L[6]	–
Cd	–	<0.1 ppm[5]	n.a.	–
Cu	–	7.9–10.6 ppm[5]	n.a.	–
Co	–	3.4–5.7 ppm[5]	n.a.	–
Ni	–	2.6–3.1 ppm[5]	n.a.	–
Hg	–	24.98–41.95 ppm[5]	40–232 μg/L[6]	–
Pb	–	14.8–20.1 ppm[5]	5.7–29.1 mg/L[6]	

[1]Detected by X-ray diffraction and energy dispersive microanalytical system-equipped electron microscope (Istituto Nazionale di Geofisica e Vulcanologia, Osservatorio Vesuviano labs in Naples, Italy) on mud (solid) and dried water separated from investigated sample. [2]Average flux values in 2013 by Aiuppa et al. (2013). [3]Flux values in 2018 by Tamburello et al. (2019). [4]From Caliro et al. (2007). [5]From Piochi et al. (2019). [6]From Valentino et al. (1999) and Valentino and Stanzione (2004). n.d., not detected; n.a., not analysed. Values for element are content in terrain/mud matrices and content dissolved in waters.

(FeS_2) coexisting with native sulfur, sulfates, and sulfides and amorphous silica found around the main fumaroles and within the mud pool (Piochi et al., 2015, 2019). Other metals found in the solfataric terrains of Pisciarelli are nickel, copper, and cobalt, at <20 ppm, whereas cadmium was not detected (Piochi et al., 2019).

At the time of sampling, the marginal water pool temperature was ∼55°C and the pH was ∼5 (**Figure 1**). To mimic the sampling site, the collected samples were initially grown in LB at pH 5 at 55°C. After serial dilutions and repeated streaking procedures on solid medium, a single colony was isolated and identified through 16S rRNA sequencing. This analysis allowed the identification of a member of the *Alicyclobacillus* genus. First representatives of the *Alicyclobacillus* genus were initially isolated from the hot springs of the Yellowstone National Park and the Hawaii Volcano National Park in the United States and from the hydrothermal waters of Solfatara (Darland and Brock, 1971; De Rosa et al., 1971); these sites share similar acidic sulfate conditions (Aiuppa et al., 2006). The genus has been named *Alicyclobacillus* since 1992 to include several isolates previously wrongly attributed to the *Bacillus* genus (Wisotzkey et al., 1992). *Alicyclobacillus* are strictly aerobic thermo-acidophilic microorganisms that produce ω-alicyclic fatty acids as major cellular fatty acids (Hippchen et al., 1981; Poralla and König, 1983) and grow optimally at temperatures between 28 and 65°C (Baumgart, 2003; Ciuffreda et al., 2015). After their discovery, a high number of enzymes have been characterized from those microbes that helped to define the molecular determinants of thermostability (Bartolucci et al., 1997; Atalah et al., 2019). To date, 19 species belonging to the genus *Alicyclobacillus* have been identified (Ying et al., 2010; Smit et al., 2011; Pornpukdeewattana et al., 2020) and are grouped into three categories depending on their growth temperature ranges: (1) in the 45–70°C range, with a temperature optimum of 65°C; (2) in the 20–65°C range, with an optimum set at 40–55°C; and (3) the range of 4–55°C with optimum growth temperatures from 35°C to 42°C (da Costa et al., 2015).

Genome Assembly and Functional Annotations

The genome was successfully sequenced and the statistics are summarized in **Table 2**. Different assembler tools were tested, and the best assembly was obtained using MEGAHIT, which comprises 48 contigs (all of them ≥1,000 bp) with a total length of ∼3 Mbp, N50 value of 128,277 bp, and an average GC content of 61.5%. To assess the genome quality, the assembly was evaluated for completeness (99.61%) and contamination (0.0%) using CheckM. Annotation of the genes in the *Alicyclobacillus* genome was carried out using RAST server and eggNOG databases. The analysis obtained from RAST revealed the presence of 3127 coding sequences, 64 RNAs, of which 62 tRNA and 2 rRNA (LSU and SSU). Moreover, the presence of 4 CRISPR arrays with 91 CRISPR spacers and 99 CRISPR repeats can be correlated to the existence of the adaptive immunity system CRISPR-Cas (**Figure 2**). The high number of CRISPR spacers can be explained by the complexity of the ecosystems from which the microorganism was isolated and by the targeting of the bacterial host from many phage (Guerrero et al., 2020).

TABLE 2 | Genome statistics of *Alicyclobacillus mali* FL18.

Genome	*Alicyclobacillus mali* FL18
Domain	Bacteria
Size (bp)	3,024,307
GC content (%)	61.5
N50 (bp)	124,285
L50	9
Number of contigs (with PEGs)	48
Number of subsystems	271
Number of coding sequences	3127
Number of RNAs	64

Interestingly, only 26% of the predicted genes was recognized by RAST and classified in 271 subsystems. The distribution of different functional groups showed a predominance of genes involved in general processes related to amino acid and carbohydrate metabolism, but several genes were also found to be involved in the biosynthesis of cofactors, vitamins, prosthetic groups, pigments, and protein metabolism (**Figure 3**). Interestingly, 78 genes were included in the subsystem of fatty acids, lipids, and isoprenoids according to the presence of a peculiar fatty acid metabolism in *Alicyclobacillus*; in particular, isoprene molecules have many physiological roles and have found several applications as fragrances, essential oils, and, most recently, biofuels (Phulara et al., 2016).

To expand the functional annotation of the isolated strain, we established orthology relationships between the sequenced genome of the *Alicyclobacillus* isolate and other annotated genomes, using the last version of the eggNOG (Huerta-Cepas et al., 2019). For this purpose, 2,507 genes were predicted and functionally annotated using KEGG pathways, Gene Ontology (GO) terms, and Clusters of Orthologous Groups of proteins (COGs), the latter listed in **Table 3**. Considering all the protein encoding genes, we found that 80.97% matched with COG functional categories and the remaining 19.03% persisted as unclassified; moreover, 42.68% of annotated proteins were clustered within the functions associated to specific metabolisms; in particular, many of them were found to be associated with amino acid transport and metabolism (E), energy production and conversion (C), and carbohydrate transport and metabolism (G). This result agrees with the annotation data obtained from RAST subsystems described above.

Taxonomic Affiliation and Phylogenetic Analysis

To shed light on the taxonomy of the new isolated *Alicyclobacillus*, we resolved to perform a phylogenetic analysis. In fact, the analysis of 16S rRNA was not straightforward to unambiguously identify the species, because, as reported in the literature, 16S rRNA sequences are very similar among members of the genus *Alicyclobacillus* (Connor et al., 2005; Goto et al., 2008). In detail, a phylogenetic tree was built using a COG approach, which is based on the analysis of universally conserved

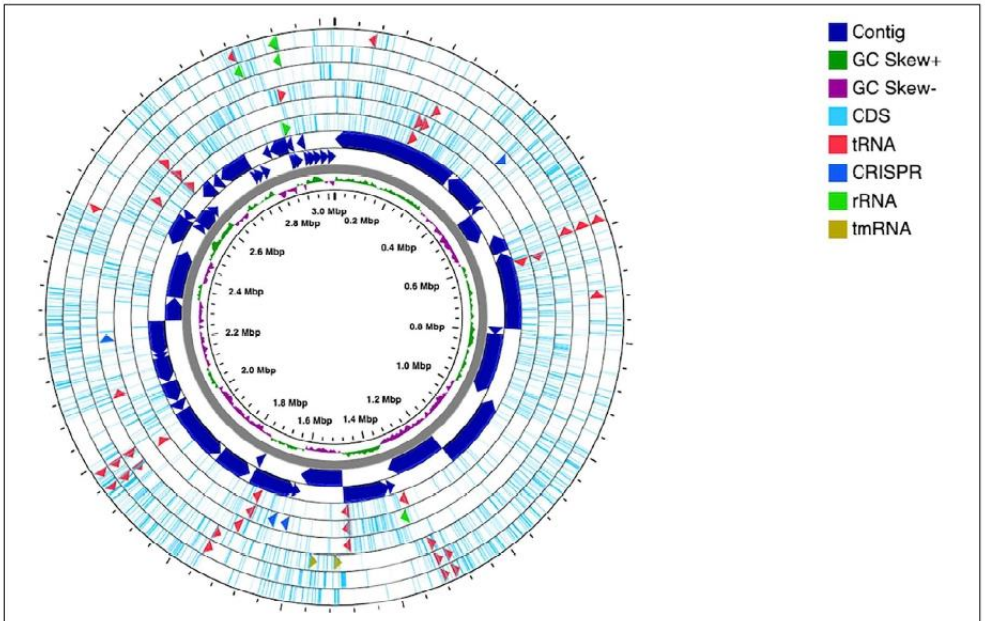

FIGURE 2 | Circular map of *Alicyclobacillus mali* FL18 genome performed using CGview Server. From the center to outside: genome size, with a ring showing the GC skews (positive values in green and the negatives in purple); the contigs, represented as dark blue arrows in a double ring to indicate the strands plus and minus; the CDSs are reported in light blue, together with tRNA (red), rRNA (green), tmRNA (dark red), and CRISPR (blue).

genes defined by COG families (**Figure 4** and **Supplementary Table 1**). This procedure is based on the notion that if small groups of proteins (at least three) from different genomes are similar to each other, they can belong to an orthologous family (Livingstone et al., 2018). By applying this method, a subset of closely related genomes was imported using RefSeq (NCBI), and the relatedness was calculated from a multiple sequence alignment (MSA) for each COG family. Afterward, the poorly aligned sections were trimmed and concatenated to reconstruct the phylogenetic tree (**Figure 4**). The isolate resulted to be closely related to *A. mali* NBRC 102425 deposited on NCBI (Accession number: NZ_BCSG01000001). Given the high evolutionary relationship between the two microbes, we turned out to assess whether the *Alicyclobacillus* isolate and *A. mali* NBRC 102425 belonged to the same species; for this reason, we calculated the average nucleotide identity (ANI) of the two whole genomes. ANI is built as an alignment-based search that gives a similarity index between two genomes; a cutoff score of >95% is indicative of members belonging to the same species (Jain et al., 2018). Indeed, the ANI value resulted to be 98.91% (**Supplementary Table 2**), strongly suggesting that the *Alicyclobacillus* isolate belonged to the species *mali*. To confirm the result, the Genome-to-Genome Distance Calculator (GGDC), was employed (Auch et al., 2010). Also, in this case, the DDH value was calculated using as genome comparison *A. mali* NBRC 102425. From the data obtained using a generalized linear model (GLM), the DDH value was 89.70% indicating that they belong to the same species (**Supplementary Table 2**). On the other hand, the value calculated to estimate the probability that the two bacteria belonged to the same subspecies was 64.81%, lower than the threshold (**Supplementary Table 2**) (Meier-Kolthoff et al., 2013). Altogether, these results suggest that the isolate is a new strain of *A. mali*. Hereinafter, the isolated strain was named *A. mali* FL18 and the complete genome sequence was deposited at NCBI GenBank under accession number JADPKZ000000000.

Comparative Genomics of *A. mali* Strains

A comparative analysis was performed to gain insights into the similarities and differences between the two *A. mali* strains (FL18 and NBRC 102425). The genome sizes of *A. mali* FL18 and *A. mali* NBRC 102425 turned out to be slightly different, i.e., 3,024,307 and 2,786,970 bp, respectively, while the GC content was comparable (61.5 and 61.9%) (**Supplementary Table 3**). The genomes of both strains were annotated using RAST server and the predicted DNA coding sequences (CDS) were 3127 and 2815 for FL18 and NBRC102425 strains, respectively (see also above).

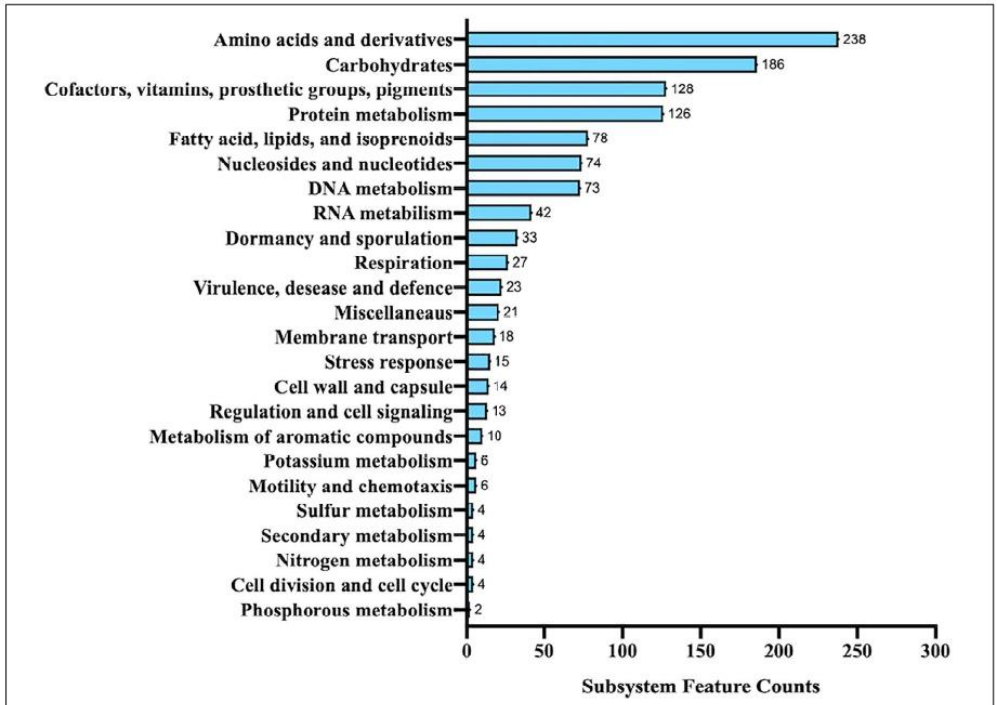

FIGURE 3 | Functional annotation of *Alicyclobacillus* isolate using RAST server.

TABLE 3 | Clusters of Orthologous Groups of proteins (COG) classification of the annotated genes in *A. mali* FL18.

	COG description	COG	Value	% of total
Cellular processes and signaling	Cell cycle control, cell division, chromosome partitioning	D	52	2.07
	Cell wall/membrane/envelope biogenesis	M	124	4.95
	Cell motility	N	59	2.35
	Post-translational modification, protein turnover, and chaperones	O	73	2.91
	Signal transduction mechanisms	T	65	2.59
	Intracellular trafficking, secretion, and vesicular transport	U	28	1.12
	Defense mechanisms	V	34	1.36
Information storage and processing	Translation, ribosomal structure, and biogenesis	J	160	6.38
	Transcription	K	197	7.86
	RNA processing and modification	A	1	0.04
	Replication, recombination, and repair	L	167	6.66
Metabolism	Energy production and conversion	C	161	6.42
	Amino acid transport and metabolism	E	251	10.01
	Nucleotide transport and metabolism	F	73	2.91
	Carbohydrate transport and metabolism	G	168	6.70
	Coenzyme transport and metabolism	H	123	4.91
	Lipid transport and metabolism	I	129	5.15
	Inorganic ion transport and metabolism	P	131	5.23
	Secondary metabolites biosynthesis, transport, and catabolism	Q	34	1.36
Poor char	Function unknown	S	477	19.03
			2507	100.00

Of these CDSs, 2386 (84.8%) and 2507 (80.2%) belonged to the COG families. In fact, **Figure 5A** shows how the distribution patterns resemble each other.

To evaluate the differences at sequence level between the strains in more detail, the bioinformatic tool Mauve was exploited (Darling et al., 2004). The graphical analysis obtained after nine steps of progressive alignments shows the presence of well conserved regions, but clearly randomly distributed (**Figure 5B**). This similarity between the two strains FL18 and NBRC 102425 was also confirmed at protein level using OrthoVenn, a web

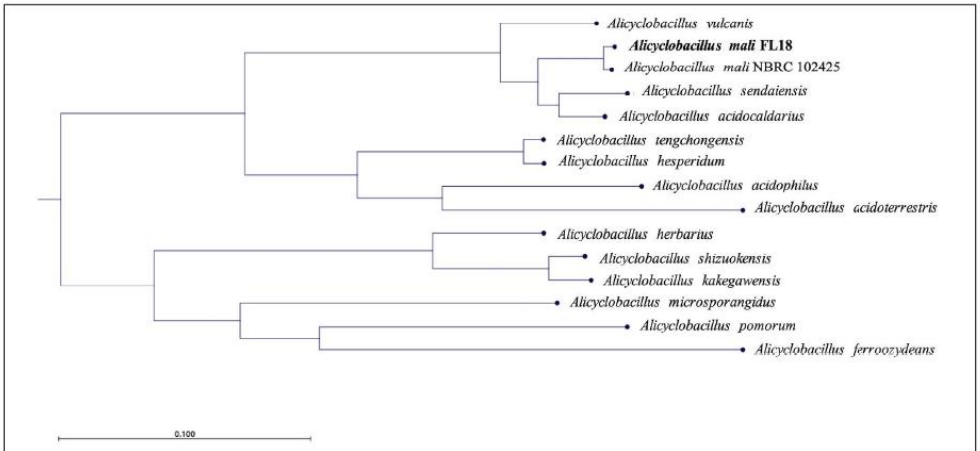

FIGURE 4 | Phylogenetic tree based on the genome comparison of *A. mali* FL18 with reference genomes publicly available at NCBI.

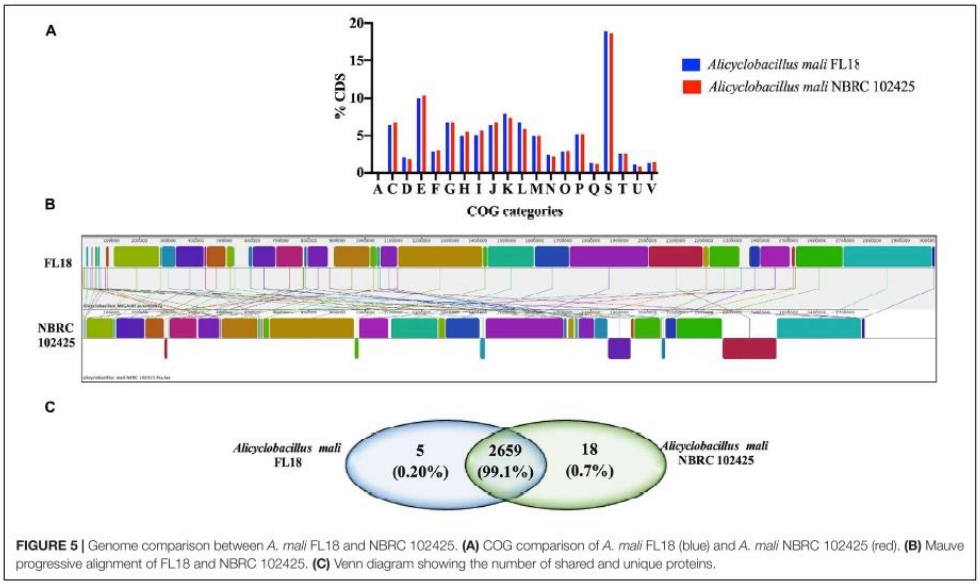

FIGURE 5 | Genome comparison between *A. mali* FL18 and NBRC 102425. **(A)** COG comparison of *A. mali* FL18 (blue) and *A. mali* NBRC 102425 (red). **(B)** Mauve progressive alignment of FL18 and NBRC 102425. **(C)** Venn diagram showing the number of shared and unique proteins.

TABLE 4 | Annotation, strand, and length of the predicted arsenic resistance genes.

Function	Strand	Length
Transcriptional regulator, ArsR family	+	239
Transcriptional regulator, ArsR family	+	96
Transcriptional regulator, ArsR family	+	312
Transcriptional regulator, ArsR family	+	112
Arsenate reductase (EC 1.20.4.4) thioredoxin-coupled, LMWP family	+	140
ArsB, Arsenite/antimonite:H^+ antiporter	+	430
ArsB, Arsenite/antimonite:H^+ antiporter	–	399
ArsA, ATPase	+	384
ArsA, ATPase	+	365
ArsM, arsenite methyltransferase SAM-dependent	+	278
ArsP, arsenite permease	+	167

platform based on the comparison and analysis of whole-genome orthologous clusters (Xu et al., 2019). In this case, the analysis confirms that 99.1% of the proteins encoded from the two *mali* strains are shared (**Figure 5C**).

Mining of the Arsenic Resistance Genes in *A. mali* FL18 Genome

Considering the presence of high concentration of arsenic in the extreme environment of Pisciarelli (Piochi et al., 2015, 2019), we attempted to identify potential genes involved in arsenic resistance using RAST annotation server (Aziz et al., 2008). In this annotation, the central component for protein identification is FIGfams, a collection of protein families classified in sets of iso-functional homologs (Aziz et al., 2008). RAST annotation highlighted the presence of an arsenal of 11 arsenic resistance genes in *A. mali* FL18 genome, encoding for putative proteins related to arsenic resistance (**Table 4**). Of these putative genes, only two were clustered (one copy of *arsB* and one copy of *arsC*), whereas the majority of putative *ars* sequences were scattered on the genome (**Figure 6**). The arsenate reductase gene *arsC* encodes an arsenate reductase belonging to the family of thioredoxin-coupled arsenate reductases, already found in other thermophilic microorganisms (Messens et al., 2002; Del Giudice et al., 2013). Additionally, two hypothetical *arsB* genes and two *arsA* genes were also found that could functionally associate in ArsAB complexes to guarantee more efficient arsenite extrusion. Moreover, four genes putatively coding for ArsR, the arsenic resistance transcriptional regulator, were detected; these proteins are metal sensor DNA-binding proteins that act as repressors, by dissociating from DNA in the presence of metal ions, thereby allowing the expression of the metal resistance genes (Wu and Rosen, 1991). As already observed in other thermophilic microorganisms, the *ars* genes are interspersed in the genome and can be controlled by multiple ArsR transcription factors (Ordóñez et al., 2005; Antonucci et al., 2017).

Regarding resistance to organoarsenicals, a gene encoding for an arsenite methyltransferase (*arsM*) and a hypothetical methylarsenite efflux permease (*arsP*) were found; their encoded proteins could contribute to transform and extrude methylated forms of arsenite (Wang et al., 2015). The increasing number of sequenced genomes and comparative studies has elucidated the distribution of arsenic determinants in

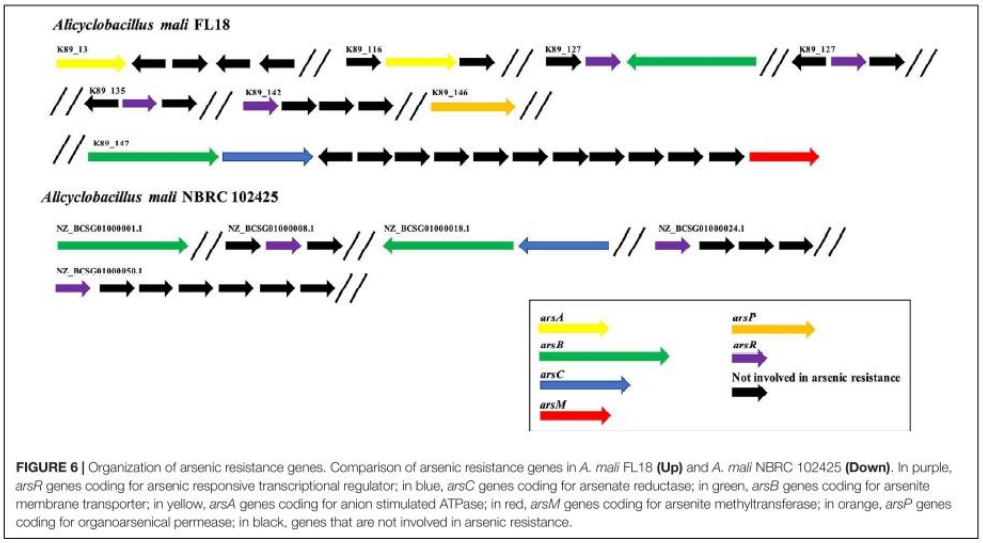

FIGURE 6 | Organization of arsenic resistance genes. Comparison of arsenic resistance genes in *A. mali* FL18 **(Up)** and *A. mali* NBRC 102425 **(Down)**. In purple, *arsR* genes coding for arsenic responsive transcriptional regulator; in blue, *arsC* genes coding for arsenate reductase; in green, *arsB* genes coding for arsenite membrane transporter; in yellow, *arsA* genes coding for anion stimulated ATPase; in red, *arsM* genes coding for arsenite methyltransferase; in orange, *arsP* genes coding for organoarsenical permease; in black, genes that are not involved in arsenic resistance.

several species, revealing that genes coding for arsenic-related processes are phylogenetically and ecologically widespread in bacteria and archaea, either mesophiles or thermophiles (Andres and Bertin, 2016). In our case study, the repertoire of arsenic resistance genes of *A. mali* FL18 was compared to that of *A. mali* NBRC 102425, and the analysis showed that our isolate has a more complex resistance system (**Figure 6**). Therefore, it is expected that it could be more tolerant to arsenic than the NBRC 102425 strain and represent a model system to unravel sophisticated arsenic resistance mechanisms.

Furthermore, to analyze the correlation between sulfur metabolism and arsenic tolerance, we looked for the presence in *A. mali* FL18 of genes responsible for reduction and fixation of sulfur into biomolecules. According to the genomic contest, neither sulfur, nor sulfide or sulfite oxygenases, nor oxidases or dehydrogenases were found, whereas genes involved in the sulfur reduction and fixation were discovered (NCBI Accession Number WP_195867833.1_2008; WP_195867500.1_1409; WP_067850267.1_2663; WP_195867655.1_935; and WP_195867654.1_934). This analysis strongly suggests that *A. mali* FL18 is not a sulfur-oxidizing bacterium; nevertheless, the correlation between arsenic and sulfur biogeochemistry in Pisciarelli hot spring could depend on the complexity of yet undiscovered microbial communities and their intricate contribution to the biogeochemical cycle of arsenic, as also demonstrated by the presence of several minerals like FeS_2 and As_4S_4.

In silico Analysis of the Heavy Metal and Antibiotic Resistance Determinants in *A. mali* FL18 Genome

Alicyclobacillus mali FL18 genome was analyzed to identify putative heavy metal and/or antibiotic resistance genes. As already reported, these resistance genes may code for efflux pump able to confer resistance to both antibiotics and metals; these elements could have co-evolved or be genetically linked on transposable genetic elements (Knapp et al., 2017). From the *in silico* analysis of *A. mali* FL18, four genes encoding multidrug resistance (MDR) efflux transporters and five genes encoding putative transcriptional regulator of the MerR or MarR families (**Figure 7** and **Table 5**) were detected. Genome analysis of *A. mali* NBRC 102425 revealed a higher number of multidrug transporters in comparison to *A. mali* FL18, suggesting that such different genetic profile is the result of adaptation to diverse environments. In *A. mali*, FL18 genes are distributed on five contigs and are not organized in canonical operon structures; only MarR transcriptional factor was found associated to putative MDR transporter in three contigs (k89_135, k89_146, and k89_147) (**Figure 7**).

The MDR efflux transporters are widespread among microbes and recognize a variety of chemically and structurally different toxic compounds as well as antibiotics and are generally regulated by several families of transcription factors that modulate the expression in response to binding of drug molecules acting as effectors (Fiorentino et al., 2007; Contursi et al., 2013).

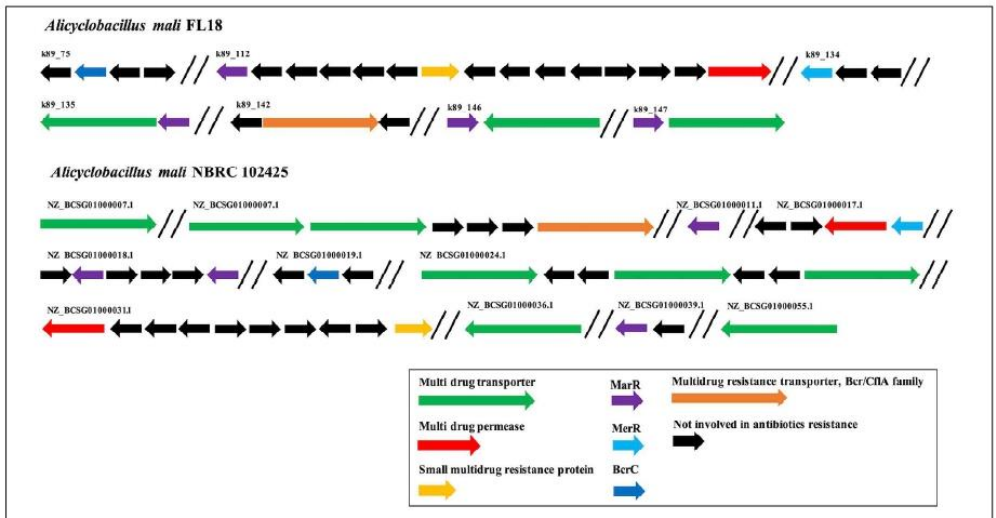

FIGURE 7 | Organization of multi-drug resistance genes. Comparison of antibiotic resistance genes in *A. mali* FL18 **(Up)** and *A. mali* NBRC 102425 **(Down)**. In purple, *MarR* genes coding for antibiotic responsive transcriptional regulator; in blue, *MerR* gene coding for mercury resistance transcriptional regulator; in green, multidrug transporter genes; in red, multidrug permease genes; in orange, small multidrug resistance protein; in black, genes that are not involved in antibiotic resistance.

TABLE 5 | Annotation, strand, and length of the predicted heavy metal and/or antibiotic resistance genes.

Function	Strand	Length
Transcriptional regulator, MerR family	+	157
Transcriptional regulator, MarR family	−	136
Transcriptional regulator, MarR family	+	129
Transcriptional regulator, MarR family	−	135
Transcriptional regulator, MarR family	−	107
Multidrug-efflux transporter, major facilitator superfamily (MFS)	+	395
Multidrug-efflux transporter, major facilitator superfamily (MFS)	−	426
Multidrug-efflux transporter, major facilitator superfamily (MFS)	−	382
Permease, multidrug efflux	+	575
Small Multidrug resistance protein	+	344
Multidrug resistance transporter, Bcr/CflA family	+	1253
Undecaprenyl-diphosphatase BcrC	+	599

These transcriptional regulators may belong to the MarR family, which is widespread across different bacterial species, probably due to horizontal gene transfer (Fiorentino et al., 2011; Beggs et al., 2020).

Noteworthy, *A. mali* FL18 contains in its genome a gene coding for a putative undecaprenyl-diphosphatase BcrC (NCBI Accession Number WP_067849704.1), which might be involved in the bacitracin resistance. In particular, BcrC in *Bacillus subtilis* is responsible for the dephosphorylation of UPP, an essential intermediate of the peptidoglycan biosynthesis. BcrC competes with bacitracin for UPP binding, promoting peptidoglycan biosynthesis, and its expression is activated by a broad range of conditions (antibiotics, ethanol, and salts) through a "damage-sensing" mechanism (Piepenbreier et al., 2020). To understand if this genetic trait was unique for *A. mali* FL18, the presence of BcrC in *A. mali* NBRC 102425 was also investigated. Surprisingly, the latter possesses the putative BcrC as well (NCBI Accession Number WP_067849704) and the two protein sequences share an identity of 100%, indicating that this gene is conserved within the species.

Assessment of Arsenic Tolerance

To perform a phenotypic characterization regarding metal ion and antibiotic tolerance, the bacterial growth of *A. mali* FL18 was tested in different media. From the analysis of growth curves, the optimal broth was found to be BAT medium, in which the doubling time was 30 min (**Supplementary Figure 1**). Afterward, *A. mali* FL18 was cultivated in BAT medium and tested for its arsenic tolerance, showing MIC values of 11 mM and 41 mM for As(V) and As(III), respectively (**Table 6**). These findings indicate that *A. mali* FL18 is an arsenic-tolerant bacterium (Altowayti et al., 2020). The MIC value toward As(III) is comparable to that of other arsenic-tolerant microorganisms, ranging from about 10 to 50 mM (Das et al., 2014; Antonucci et al., 2017). Interestingly, the high MIC toward As(III) is close to that of two As(III) oxidizing strains of *Bacillus* and *Geobacillus* (16 and 47 mM, respectively) isolated from contaminated soils of West Bengal (Majumder et al., 2013).

TABLE 6 | *Alicyclobacillus mali* FL18 tolerance to heavy metal ions.

MIC value	
As(V)	11 mM
As(III)	41 mM
Cd(II)	0.8 μM
Cu(II)	0.5 mM
Co(II)	3 mM
Ni(II)	30 mM
Hg(II)	17 μM

The observed As(III) tolerance can be explained by the occurrence of MDR transporters and by the presence of a higher number of genes specific for arsenite (arsenite methyltransferase, arsenite efflux systems, and organoarsenical permease) rather than arsenate resistance (arsenate reductase) (**Figures 6**, **7**).

To better compare the effect on cellular growth of As(V) and As(III), *A. mali* FL18 was cultivated at half of the MIC value (6 mM and 20 mM, respectively). As expected, a significant decrease in the duplication time was observed (**Figure 8**); indeed, the generation time was increased from 30 to 60 min in the presence of 20 mM As(III) and 6 mM As(V), respectively. The longer replication time can be traced back to arsenic toxicity, the effects of oxidative stress to the cells, and/or the time needed to activate the resistance pathways.

Furthermore, in order to verify if *A. mali* FL18 was able to perform arsenic bioconversion, a qualitative colorimetric assay was used to test the growth in the presence of arsenic ions.

When *A. mali* FL18 was cultivated in the presence of 20 mM As(III), a bright yellow precipitate was obtained in both supernatants and cells, suggesting that As(III) is not converted to As(V). Conversely, when *A. mali* FL18 was grown in the presence of 5 mM As(V), a color change was observed (**Figure 9**). Although quantitative and/or biochemical data are required, this experiment indicates that *A. mali* FL18 reduces As(V) and does not oxidize As(III), compatible with our genomic analysis, which highlighted the presence of a putative arsenate reductase but not of an arsenite oxidase (**Table 4**).

Heavy Metals Tolerance in *A. mali* FL18

Solfataric environments are characterized by the coexistence of various toxic substances; in particular, at Pisciarelli, high levels of CO_2, H_2S, and NH_4 coexist with As, Hg, Fe, Be, Ni, Co, and Cu (Aiuppa et al., 2006; Cardellini et al., 2017; Piochi et al., 2019). **Table 6** shows that *A. mali* FL18 has a heterogeneous profile of metal tolerance; in particular, it exhibits high MIC values for nickel, cobalt, and mercury (30 mM, 3 mM, and 17 μM, respectively, **Table 6**) (Sharma and Shukla, 2020), but low for copper (0.5 mM). Regarding cadmium, the MIC value (0.8 μM) is indicative of an almost null tolerance, in agreement with the fact that the genome does not apparently contain any cadmium resistance gene and supporting the idea that, differently from other thermophilic microorganisms, the arsenic resistance system is specific and selective (Antonucci et al., 2018). Notably, Cd is virtually absent at Pisciarelli (Piochi et al., 2019).

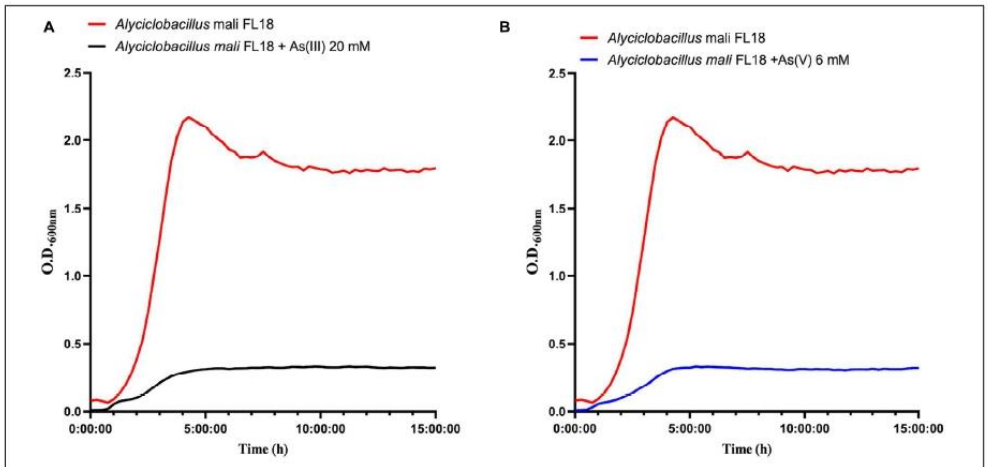

FIGURE 8 | Growth curves of *A. mali* FL18 in the presence of 20 mM As(III) **(A)** and 6 mM As(V) **(B)**. The red curve is the As-free control growth.

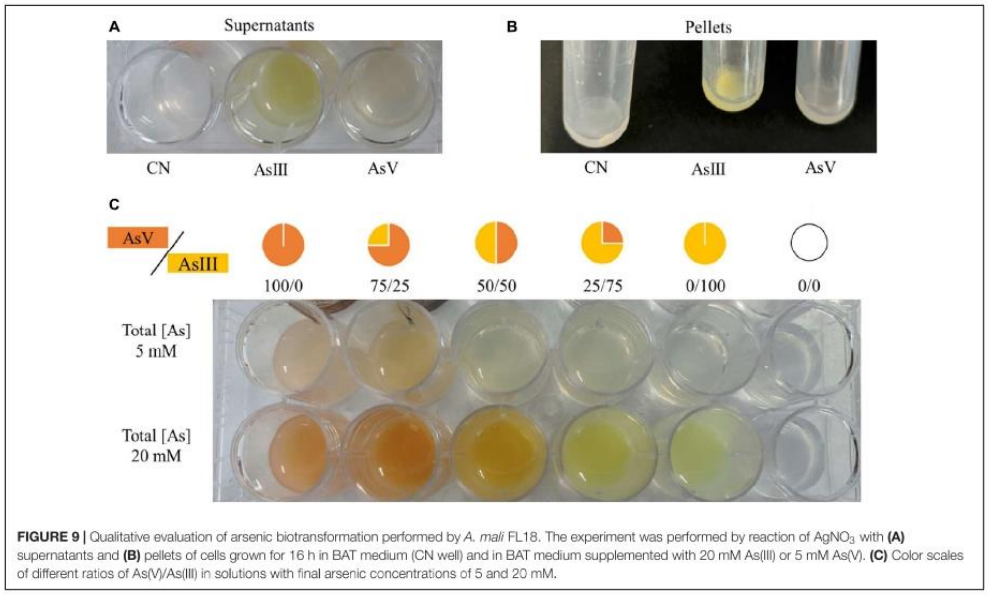

FIGURE 9 | Qualitative evaluation of arsenic biotransformation performed by *A. mali* FL18. The experiment was performed by reaction of $AgNO_3$ with **(A)** supernatants and **(B)** pellets of cells grown for 16 h in BAT medium (CN well) and in BAT medium supplemented with 20 mM As(III) or 5 mM As(V). **(C)** Color scales of different ratios of As(V)/As(III) in solutions with final arsenic concentrations of 5 and 20 mM.

Antibiotic Susceptibility in *A. mali* FL18

Since the correlation between heavy metals and antibiotics is a well-known complex interaction (Ye et al., 2017) and due to the presence in the genome of genes coding for antibiotic resistance, we also analyzed the resistance of *A. mali* FL18 to antibiotics, determining the MIC values (**Table 7**).

With the aim to investigate susceptibility to antibiotics presenting diverse mechanisms of action, antibiotics belonging to different classes were chosen, particularly aminoglycosides

TABLE 7 | *Alicyclobacillus mali* FL18 susceptibility to antibiotics.

MIC value	
Ampicillin	20 μg/ml
Bacitracin	700 μg/ml
Chloramphenicol	<0.5 μg/ml
Ciprofloxacin	>1 mg/ml
Erythromycin	70 μg/ml
Kanamycin	80 μg/ml
Streptomycin	70 μg/ml
Tetracycline	<0.5 μg/ml
Vancomycin	1 μg/ml

(kanamycin and streptomycin), chloramphenicol, macrolides (erythromycin), and tetracyclines that inhibit protein synthesis; fluoroquinolones (ciprofloxacin) that inhibit the DNA replication; and β-lactams (ampicillin), glycopeptides (vancomycin), and antibiotic polypeptides (bacitracin) that inhibit the peptidoglycan synthesis in Gram-positive bacteria (Kapoor et al., 2017).

Interestingly, *A. mali* FL18 became resistant to all the antibiotics tested, except to chloramphenicol and tetracycline. The MIC values determined against ampicillin, erythromycin, kanamycin, streptomycin, and vancomycin were comparable to those of other antibiotic-resistant bacteria, as well as those relative to bacitracin (700 μg/ml) and ciprofloxacin (up to 1 mg/ml) (D'Aimmo et al., 2007; Minarini et al., 2012; Bulut et al., 2020). Besides the putative gene coding for BcrC and possibly responsible for bacitracin resistance (see above), it can be hypothesized that the expression of several multidrug-efflux transporters (**Table 5**) is responsible for the resistance to the other tested antibiotics. To the best of our knowledge, the co-occurrence of heavy metals and antibiotic resistance genes is a well-known mechanism in multidrug-resistant microorganisms such as *Pseudomonas aeruginosa* and *E. coli* (Nguyen et al., 2019), but not yet reported in extremophiles (Najar et al., 2020).

On the other hand, the resistance to ciprofloxacin is generally associated with mutations in the quinolone resistance-determining regions (QRDR) of *gyr*A and *par*C genes, respectively, coding for the subunit A of the DNA gyrase and the topoisomerase IV (Werner et al., 2010; Minarini et al., 2012). In particular, mutations in *gyr*A are able to confer resistance to ciprofloxacin, while mutations in *par*C can further improve the resistance (Hamouda and Amyes, 2004). Interestingly, in *A. mali* FL18, the subunit A of the DNA gyrase has two amino acid substitutions in correspondence to the QRDR, known to be responsible for the acquired resistance in *Enterobacteriaceae*, including *E. coli* (Minarini et al., 2012) (**Supplementary Figure 2**).

CONCLUSION

Microbial bioremediation of toxic metals from the environment is a hot topic in the field of clean-up of contaminated sites; in this context, the exploitation of microbes flourishing in primordial niches rich in such toxic metalloids are very good candidates since they have been subjected to natural selection for a long time.

In this work, we identified and characterized a novel thermo-acidophilic bacterium, *A. mali* FL18, from a geothermal environment naturally rich in arsenic. Genome mining performed to target genes involved in metal detoxification revealed the presence of signatures typical of arsenic-tolerant microbes and of many multidrug efflux systems suggesting its ability to tolerate different toxic compounds.

Interestingly, the comparison of the repertoire of metal/drug resistance genes of our isolate with that of *A. mali* NBRC 102425 deposited on NCBI showed a higher number of arsenic resistance genes in *A. mali* FL18, mirroring a better potential in arsenic tolerance and suggesting that their different genetic profile is the result of their adaptation to specific niches. Analyses performed to test the capability of *A. mali* FL18 to tolerate heavy metals and different antibiotics confirmed its tolerance to a wide variety of different toxic compounds.

Altogether, these molecular and phenotypical features indicate that *A. mali* FL18 represents a suitable platform for further investigation either to address bioremediation of heavy-metal pollution and to identify new thermostable proteins/enzymes for industrial applications.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MA, GG, and RP: data curation. MA, GG, RP, MP, AM, SB, and GF: investigation. MA, RP, GG, MP, AM, and GF: methodology. DL, PC, MP, SB, and GF: supervision. MA, GG, RP, MP, AM, SB, and GF: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.639697/full#supplementary-material

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Chapter [5]: Extremophiles, a nifty tool to face environmental pollution: from exploitation of metabolism to genome engineering

Extremophilic microorganisms are a rich source of naturally tailored biomolecules for various fields of application.
Especially thermophiles are of high potential for versatile industrial and environmental processes. In fact, their ability to process waste biomasses and/or chemical pollutants is considered a new opportunity for bio-catalysis and bio-transformations, which will not only contribute to the protection of the environment, but also to generate biotechnological products with high added value. In this context, there is an increasing need to engineer these microorganisms in order to optimise their performance, and in fact, for this purpose, new genetic tools based on more recent technological advances have been made available in recent years. Regarding in particular *T. thermophilus*, the thermophilic model organism used in our laboratory, the new thermo-adapted CRISPR-Cas9 editing system (Ref [183-185] in the review) opens the way to whatever manipulation of its genome, including the possibility to realize a stable, plasmid-free whole-cell biosensor for arsenic.
This chapter represents the closing of a circle regarding the exploitation of thermophiles: Chapter [2] presented their biotechnological applications in relation to heavy metals, this chapter recalls these concepts, and also explores the resistance mechanisms of thermophiles to other polluting compounds, as well as their employment in bioremediation processes.

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Review

Extremophiles, a Nifty Tool to Face Environmental Pollution: From Exploitation of Metabolism to Genome Engineering

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Abstract: Extremophiles are microorganisms that populate habitats considered inhospitable from an anthropocentric point of view and are able to tolerate harsh conditions such as high temperatures, extreme pHs, high concentrations of salts, toxic organic substances, and/or heavy metals. These microorganisms have been broadly studied in the last 30 years and represent precious sources of biomolecules and bioprocesses for many biotechnological applications; in this context, scientific efforts have been focused on the employment of extremophilic microbes and their metabolic pathways to develop biomonitoring and bioremediation strategies to face environmental pollution, as well as to improve biorefineries for the conversion of biomasses into various chemical compounds. This review gives an overview on the peculiar metabolic features of certain extremophilic microorganisms, with a main focus on thermophiles, which make them attractive for biotechnological applications in the field of environmental remediation; moreover, it sheds light on updated genetic systems (also those based on the CRISPR-Cas tool), which expand the potentialities of these microorganisms to be genetically manipulated for various biotechnological purposes.

Keywords: extremophiles; environmental pollution; heavy-metal resistance; aromatic-compounds; bioremediation; biosensors; genome-engineering; CRISPR-Cas

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1. Introduction

Natural environments on Earth display the most miscellaneous life conditions, and microorganisms are among the few entities that are able to grow in very extreme and inhospitable habitats. Hot springs, volcanic areas, polar regions, saline-alkaline, or acidic lakes and deep-see hydrothermal vents are some examples of natural environments that show temperature, salt concentration, pH, and pressure conditions very harsh for almost all forms of life [1].

Extremophiles are microorganisms that can live in these kinds of natural niches, and based on the conditions in which they thrive, they can be grouped in: acidophiles/alkaliphiles that grow at acid or alkaline pHs, halophiles that can live at high salt concentrations, piezophiles that prosper in high pressure conditions, metallophiles that are able to thrive in presence of metals/heavy metals, psychrophiles which live at low temperatures, and thermophiles/hyperthermophiles that grow at elevated temperatures [2]. Moreover, as a result of anthropogenic activities, some microbes adapted to flourish in polluted environments such as industrial wastewaters and contaminated soils characterized by the presence of toxic substances like pesticides, heavy metals, and different chemicals [3,4]. Therefore, extremophiles possess peculiar biological molecules and metabolic pathways that allow them to face multiple environmental stresses, sometimes simultaneously. For example,

enzymes of thermophiles (called thermozymes), in comparison to their mesophilic counterparts, maintain their activity and their folding at higher temperatures due to a more compact hydrophobic core, and a better distribution of hydrogen bonds and salt bridges at the protein surface [5]. At the same time thermozymes are also stable in the presence of organic solvents, denaturing agents, and high salinity, and more resistant to proteolysis, mirroring the niches where they are found. So far, extremophiles have increasingly received attention for their biotechnological significance and for industrial purposes; the study of these peculiar microorganisms, their metabolisms and catalysts makes possible to develop bioremediation technologies and bio-based energy processes.

In the last years, as a consequence of environmental pollution, global warming, and depletion of non-renewable sources and with the push of the 2030 Agenda for Sustainable Development drawn up by the United Nations [6], many research efforts have been focused either on the optimization of green and sustainable industrial processes (biorefineries), and on the setup of biotechnological methods to monitor and remove pollutants from the environment (biomonitoring and bioremediation, respectively) [7,8]. In this context, investigation on the biology, ecology, and physiology of microorganisms is a necessary prerequisite to set up white and green biotechnologies [9] in the field of industrial processes, energy generation [10], prevention of environmental pollution by detection and/or removal of contaminants [11], and production of biopolymers from renewable resources [12,13]. For example, thermophilic microorganisms can find applications to reduce pollution in industrial wastewaters that are often characterized by higher temperatures and highly dissolved heavy metals [14,15]. Moreover, thermophiles are more advantageous than mesophiles in biorefineries which require high-temperature steps [16].

This review analyses the metabolic strategies adopted by extremophilic microorganisms, with major emphasis on thermophilic ones, to face three classes of compounds with high impact on environmental pollution: heavy metals, organic compounds, and lignocellulosic biomasses, as well as their exploitation for application in bioremediation, biosensing, and biorefinery. Moreover, it provides updates regarding available genetic systems to engineer these microorganisms, in order to use them as platforms for metabolic engineering and production of valuable compounds.

2. Heavy Metals

The term "heavy metals" is widely referred to a group of metals and metalloids associated with potential toxicity or ecotoxicity. Generally, these metals possess relatively high densities, atomic weights, or atomic numbers. The criteria used for this classification vary, depending on the author and the context [17]; a recent paper reported by the *International Journal of Environmental Research and Public Health*, proposed to refer to them as "potentially toxic elements" [18]. Heavy metals are among the most persistent and toxic pollutants in the environment (Figure 1); they are non-biodegradable, and even in small concentrations, can threaten human and environmental health [19]. Heavy metals naturally occur in soils, rocks, sediments, air, and waters and microbial communities affect their speciation and mobility in the environment, because they are actively involved in metal geochemical cycles [20]. In traces, several heavy metals are essential for life; almost half of all enzymes require the presence of a metal atom to function [21]. Some of them as iron, copper, nickel, manganese, and zinc play key roles as functional centers in proteins and enzymes (i.e., metalloproteins) allowing biological transformations that are exceptionally unlikely to proceed spontaneously [22], as manganese in manganese-peroxidases or copper in laccases [23]; in fact these metallozymes are often employed as industrial biocatalysts (see Section 4.1, Lignin degrading thermozymes). The uncontrolled urbanization and the anthropogenic activities have much altered metal amounts in the environment; in fact, heavy metals are released from mining activities and industrial wastes, vehicle emissions, microplastics floating in the world's oceans or they come from common devices as lead-acid batteries, fertilizers, paints [24–26]. On the other hand, their use is expected to increase over time,

Int. J. Environ. Res. Public Health **2021**, *18*, 5228

since many heavy metals, like copper or nickel, have been identified by the European Commission as critical raw materials for the transition to green energy technologies [27,28].

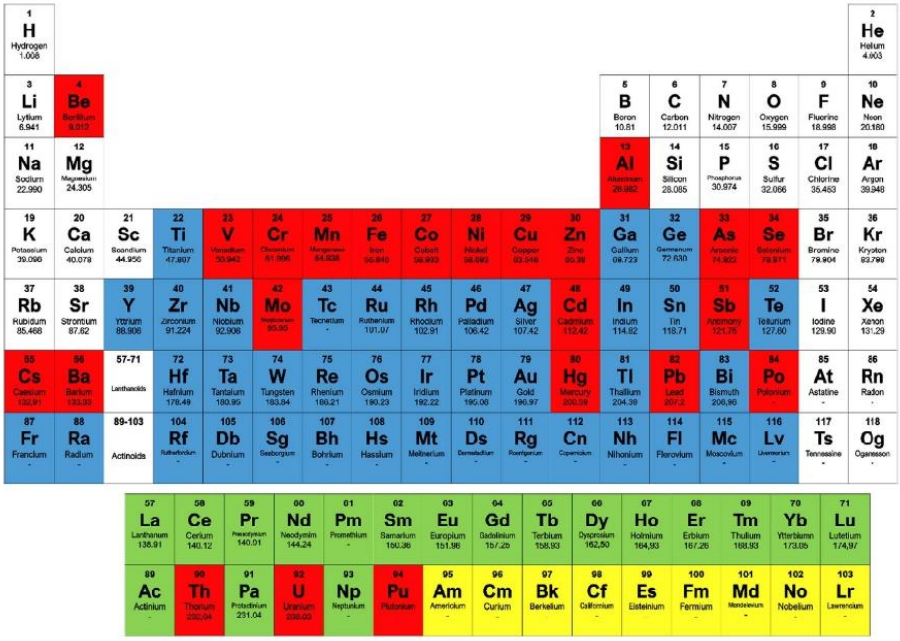

Figure 1. Periodic table of elements. Metals/metalloids are highlighted on the basis of the main characteristic that define them as "heavy": density > 5 g/cm^3 (**blue**); toxic (**red**); rare (**green**); synthetic (**yellow**).

Microorganisms have evolved resistance systems to cope with these toxic metals that usually rely on a balance between uptake and efflux processes [29]; many of these systems are also common in mesophiles, but in thermophilic Bacteria/Archaea they can present peculiar features [30]. The comprehension of the heavy metals resistance systems in thermophiles is increasingly supported by genome analyses, which allow to individuate their putative molecular determinants [31,32]. To date, at least four mechanisms of heavy metal resistance have been described: extracellular barrier; active transport of metal ions (efflux); enzymatic reduction of metal ions; intracellular sequestration [33–39]. Some bacteria are able to form complexes or chelates with extracellular polymers that reduce the permeability of metals [40]. However, heavy metals can escape this system and enter the cell thanks to the uptake systems of elements essential for life, for example, arsenic enters the cell via the phosphate or the glucose transporters [41]. Usually, the resistance to heavy metals is due to the coordinated work of intracellular enzymatic oxido-reduction and heavy metal efflux systems which generally consuming ATP, pushes the toxic metal outside the cell [42,43]. The resistance genes are usually organized in operons that also guarantee the expression of a transcription factor that regulates the whole system. Regarding the intracellular sequestration of heavy metals, the general mechanism foresees that some proteins, rich in cysteine residues, form complexes with the metals by exploiting the thiol groups [30].

Sometimes the same microorganism owns more metal resistance mechanisms; for example, *Escherichia coli* possesses either a copper active transport system (CopA) and another system based on multicopper oxidases (CueO) and CusCFBA transport system for periplasmic copper detoxification [23,44]. These resistance mechanisms are often activated as stress response [45,46]; understanding their underpinning molecular basis is crucial for application in the environmental monitoring of metal contamination (biosensing) and/or to set up bioremediation processes [14,47,48].

Biometallurgy is the branch of biotechnology that exploits the interaction between microorganisms (or their components) and metals or metal-bearing minerals (Figure 2) [49]. It includes microbial processes as metal biosorption, bioaccumulation or biomining (described below); these processes play a crucial role, on one hand, in the supply of critical raw materials, because can offer eco-efficient alternatives to classical pyro- or hydrometallurgical processes [50,51], and on the other hand in the set-up of strategies for metal biomonitoring and bioremediation (Table 1). In this context, the exploitation of thermophiles offers several advantages related to their ability to survive under harsh conditions and to degrade recalcitrant mineral species. Furthermore, in principle they could be successfully used in situ for metal bioremediation and/or biorecovery in any environment [52].

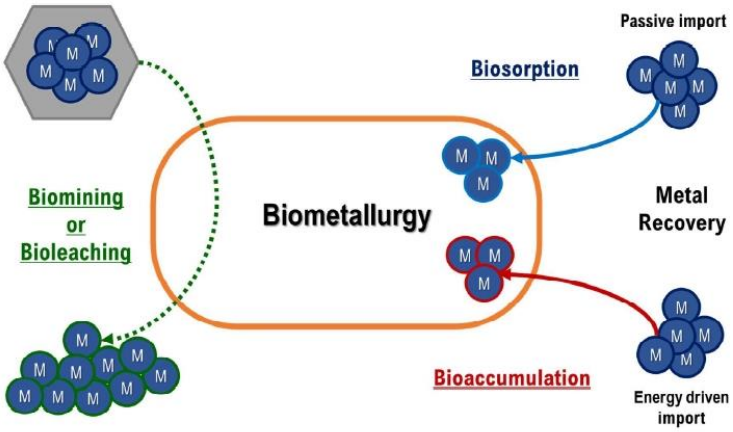

Figure 2. Schematic representation of metal bioprocesses in biometallurgy. Metals (blue circles) can be extracted from ores through biomining/bioleaching (green arrow) and they can be recovered into the cell by passive import (biosorption—blue arrow) or energy driven transport (bioaccumulation—red arrow).

Table 1. Examples of thermophiles exploited in biometallurgy.

Heavy Metals Tolerant Thermophiles				
Application	**Target**	**Microorganism**	**Temperature**	**Ref.**
Bioleaching	Cu, Zn, Ni, Cd, Al, Cr, Pb	Consortium of *Sulfobacillus thermosulfidooxidans* and *Thermoplasma acidophilum*	45 °C	[53]
Biosorption	Ag, Cd, Co, Cr, Cu, Fe, Pb, Zn	*Geobacillus thermodenitrificans*	60 °C	[3]
Biomineralization and Bioaccumulation	Eu	*T. scotoductus* SA-01	65 °C	[54]
Biosensing	Cd, As	*T. thermophilus* HB27	70 °C	[55]

Table 1. *Cont.*

Heavy Metals Tolerant Thermophiles				
Application	**Target**	**Microorganism**	**Temperature**	**Ref.**
Biosensing	Ni, Zn, Co, Hg, Mn, Cr, Cu, Fe, Cd	*A. amylolyticus*	60 °C	[56]
Biosorption	Cd, Cu, Co, Mn	*Geobacillus thermodenitrificans* and *A. amylolyticus*	60 °C	[57]
Biosorption	Cd	*Geobacillus stearothemophilus*	60 °C	[58]
Bioaccumulation	Cd, Cu, Ni, Mn, Zn	*Geobacillus toebii* subsp. *Decanicus* *Geobacillus thermoleovorans* subsp. *stromboliensis*	60 °C	[59]
Biosorption	U, Th	*B. cereus* SO-14	65 °C	[60]
Biosensing	As, Cd, Hg, Pb	*Acidibacillus ferrooxidans*	45 °C	[61]

The possibility to combine metal biorecovery with bioremediation represents an intriguing challenge to reduce process costs: microorganisms can recover metals from polluted sites, contemporarily reducing pollution, and producing valuable elements [62]. The microbial pathways that can be exploited to remove toxic metals from an environment are those related to biosorption/bioaccumulation; they consist into the ability of microorganisms to sequester heavy metals on the cell surface or intracellularly. In particular, the term "biosorption" is referred to passive processes that follow a kinetic equilibrium, while "bioaccumulation" to energy driven processes which require active metabolism [63]. For example, heavy metals metabolic pathways have been widely investigated within the *Geobacillus* genus, since many members of this species are highly tolerant to various heavy metals (As, Ag, Cd, Co, Cr, Cu, Fe, Pb, U, Zn) [57,59,64]. In particular, their biosorption and bioaccumulation mechanisms have been analyzed and applied on environmental samples to remove unwanted metals [3,58,59].

On the other hand, biomining (called "bioleaching" if metals are solubilized during the process) consists in the ability of microorganisms to extract and recover metals from ores and waste concentrates [65–67]. Several microbial species, as *Sulfobacillus* sp. and *Ferroplasma* sp., are well known for their ability to solubilize Fe(II) [31,68]; usually they are acidophilic chemolithotrophs (autotrophs or mixotrophs) presenting iron and/or sulfur oxidizing pathways [69]. In some cases, they are used as part of microbial *consortia*, which can perform the bioleaching of different metals simultaneously [53].

Microorganisms able to extract and accumulate metals from ores or geothermal sources are very interesting for their potential application in the biorecovery of rare-earth metals, for example, the Europium (Eu), which is widely used for the production of modern devices (solar cells, mobile phones and computers, biomedical instruments); *Thermus scotoductus* SA-01 can survive in the presence of high levels (up to 1 mM) of Eu, a concentration hundred times higher than that typically found in the environment and is able to extract and accumulate it from geothermal fluids [54]. The biorecovery can be useful also for monitoring metals at low concentration in the environment: Özdemir S. and co-workers [60] set up a preconcentration method with *Bacillus cereus* SO-14 to increase sensitivity in the detection of U(VI) and Th(IV) by ICP-OES (Inductively Coupled Plasma—Optical Emission Spectrometry).

Microorganisms have also been exploited for biomonitoring as *whole-cell* biosensors. In *Thermus thermophilus* HB27, the arsenic responsive transcriptional repressor *Tt*SmtB regulates the expression of the arsenic efflux protein *Tt*ArsX, in particular, *Tt*SmtB responds to variation of Cd(II), As(III) and As(V) concentrations [68,69]; therefore, Antonucci and co-workers engineered *T. thermophilus* to express a reporter gene from the *Ttars*X promoter [55].

In the set-up of systems for metal biomonitoring, it is also possible to follow a decrease of enzymatic activity as a toxicological indicator of heavy metals: Poli and co-workers observed a decrease in the α-amylase activity of *Anoxybacillus amylolyticus*, in the presence

of heavy metals [56]. In contrast, Shih-Hung and co-workers followed the inhibition of the iron-oxidizing activity of an *Acidibacillus ferrooxidans* strain [61].

In the fields of heavy metals bioremediation and biomonitoring many thermophilic oxidoreductases have also been characterized. For example, quinone oxidoreductase, chromate reductase, and superoxide dismutase from different *Anoxibacillus* species have been employed for Pb and Cr bioremoval [70,71]; moreover, the arsenate reductase from *T. thermophilus* HB27 (*Tt*ArsC) has been exploited as the biological recognition element for the development of different arsenic biosensors [72–74].

3. Organic Pollutants

Organic pollutants are a wide class of chemically different organic compounds released in the environment as toxic wastes [75]. They originate from domestic sewage, urban run-off, industrial effluents, and agricultural wastewater and include pesticides, fertilizers, hydrocarbons, phenols, plasticizers, biphenyls, detergents, oils, greases, and pharmaceuticals [76]. Therefore, the organic pollutants are a very heterogeneous group: the main constituents of the persistent organic pollutants (POPs) are organochlorinated pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCB), dioxins, and dibenzofurans; they are persistent because they remain intact in the environment for extended periods (years or decades in soil/sediment) [77,78]. These compounds are released in air and soil and even though they are scarcely soluble in water, can be biomagnified in living organisms and cause adverse effects to human health [79]. Among the most common environmental pollutants of the marine environment, there are petroleum hydrocarbons that contaminate the sea through natural oil spills, like reservoirs and volcanic processes in the deep ocean, and artificial oil spills, as oil tanker accidents, oil transportation processes, or oil refineries. In most cases, this last process represents the primary way to contaminate the sea with crude oil (Figure 3) [80,81].

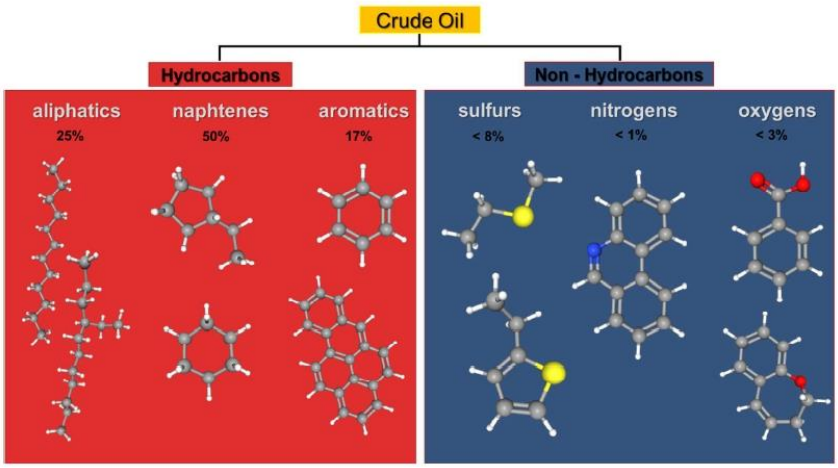

Figure 3. Schematic representation of organic compounds in crude oil. Atoms are reported in grey (C), white (H), yellow (S), blue (N) and red (O).

Microbial activities on anthropogenic organic compounds usually arise from evolution of previously existing enzymes and metabolic pathways. Microorganisms have evolved effective catalysts for detoxification of toxic compounds, as result of a selective pressure [82]. Generally, toxic compounds are converted into metabolites entering central metabolic

pathways: for example, pathways responsible for the biodegradation of aliphatic and alicyclic carboxylic acids include β-oxidation, combined α- and β-oxidation, and aromatization pathways [83]. In *Sulfolobus solfataricus* members of the multiple antibiotic resistance regulators family (MarR-family) are involved in detoxification of aromatic compounds, as benzaldehyde and salicylate [84,85]. Aromatic hydrocarbon dioxygenases, belonging to the large family of Rieske non-heme iron oxygenases (ROHs), catalyze the initial reaction in the bacterial biodegradation of a diverse array of aromatic and polyaromatic hydrocarbons, aromatic acids, chlorinated aromatics, and heterocyclic aromatic compounds [86]. They are attractive in biotechnology for bioremediation as well as for the production of industrially and medically important chiral chemicals; for example, toluene dioxygenase catalyze the oxidation of benzene to benzene *cis*-diol [87], and many thermophilic bacteria distributed mainly among *Chloroflexi*, *Deinococcus–Thermus*, and *Firmicutes* have been identified as sources of these appealing enzymes [88].

Bioremediation of toxic compounds represents an effective and sustainable technology, compared with physical and chemical remediation technologies, based on microbial activities that in an ideal bioprocess degrade all the substances to CO_2 and H_2O (complete mineralization) [89]. In crude-oil bioremediation processes, the exploitation of thermophiles can be considered an optimal choice; in fact, at higher temperature there is a decrease in the oil viscosity that increases the diffusion rates of organic compounds making them more accessible to microbial degradation [90]. Since the degradation activity can be often substrate-specific, biodegradation processes can be optimized using microbial consortia, thus expanding the spectrum of action [91,92]; for example, two strains of *Geobacillus jurassicus* and *Geobacillus subterraneus*, isolated from the Dagang high temperature oil field in China, can grow on benzoate but not phenol; therefore, to degrade crude oil, they need the presence of complementary phenol degrading activities (in this specific case a *G. stearothermophilus*, which can use phenol but not benzoate) [93].

In addition to biodegradation processes (Table 2), thermophiles also produce macromolecules that can be considered useful for the bioremediation of organic pollutants: *Bacillus licheniformis* and *Anaerophaga thermohalophila* have been characterized for the production, under anaerobic conditions, of low molecular weight peptides, which are surface-active compounds, exploitable as biosurfactants for oil removal [94].

Table 2. Examples of thermophiles exploited in bioremediation processes of organic compounds.

Organic Compounds Degrading Thermophiles				
Bioprocess	**Target**	**Microorganisms**	**Temperature**	**Ref.**
Biodegradation	Crude oil	Consortium of *Bacillus*, *Geobacillus* and *Clostridium*	55 °C	[91]
Biodegradation	Hydrocarbons	*Geobacillus pallidus*	30–70 °C	[4]
Biofilter	Volatile Organic Compounds (VOCs)	Consortium of 25 genera belonging to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Flavobacteriia, Sphingobacteriia, and Bacilli classes	50–60 °C	[92]
Biodegradation	Phenolic compounds	*Bacillus thermoleovorans* sp. A2	65 °C	[95]
Biodegradation	Hydrocarbons	Consortium of *Geobacillus* and *Thermoattinomyces* spp.	60 °C	[93]

The substrate specificity and the stability of detoxifying thermozymes make them also exploitable as recognition elements of biosensors, especially those which require electrochemical detection; for example, the haloacid dehalogenase, L-HAD_{ST} of *Sulfolobus tokodaii* was immobilized an N-hydroxysuccinimidyl Sepharose resin and used for the detection of halogenated organic compounds, retaining 70% of its initial activity after storage at 4 °C for 6 months [95,96].

Toxic Dyes

Industrialization has represented one of the main causes of water pollution. Wastewaters can be rich in recalcitrant, mutagen and carcinogenic compounds [97]. Dyes are a class of very toxic pollutants that are released in the wastewaters of textile manufacturing. These recalcitrant compounds change both the pH and the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in aquatic ecosystems [98,99]. The dyes are classified on the basis of their chemical structure or industrial uses. The most employed dyes are acid dye, synthetic dye, and direct dye. Even if they can are complex organic molecules, each dye has a characteristic chromophore: for example, the acid and synthetic azo dyes are typical for their azo linkage (–N = N–), the central chromophore of the anthraquinone dyes derives from the oxidation of anthracene, the indigoid dyes derive from indoles [100].

There are several technologies for colored wastewater remediation: physical, chemical, and biological. The physical treatments include screening, coagulation, precipitation, adsorption and membrane filtration; the chemical treatments comprise coagulation–flocculation, oxidation, ozonation, Fenton oxidation, photocatalytic oxidation, ion exchange, and electrochemical treatments; the biological methods are aerobic, anaerobic, and anaerobic–aerobic treatments in which the contaminated organic compounds are converted into safe and stable compounds [101,102]. Each technology has advantages and inconveniences; in fact, to date the typical method for wastewater remediation of colored waters is physical–chemical flocculation combined with biological treatment [101,102].

Interestingly, the analysis of microbial communities of these waters revealed the occurrence of several bacteria able to decompose the dyes: they possess intra/extra cellular oxidoreductases, such as dye decoloration peroxidases (DyP), laccases, azoreductases [103]. Many laccases and azoreductases have been characterized in several thermophiles like *T. thermophilus*, *Geobacillus, Anoxibacillus, Thermosediminibacter* species, and others (Table 3). Their enzymes are expected to be more stable to extreme temperatures and pHs in comparison to those of mesophilic bacteria.

Table 3. Examples of dye-decolorizing thermophiles.

Microorganisms	Substrates	Ref.
Anoxybacillus pushchinoensis, Anoxybacillus kamchatkensis and Anoxybacillus flavithermus	Reactive Black 5	[104]
Anoxybacillus sp.	Congo red	[105]
G. stearothermophilus	Remazol Brilliant Blue R, Methyl Orange, Malachite Green (MG) and Indigo Carmine	[106]
T. thermophilus HB27	Dye orange, Acid red dye, green dye, naphthol brilliant blue, Remazol brilliant blue, congo red	[107]
T. thermophilus SG0.5JP17-16	Congo Red, Reactive Black B and Reactive Black WNN, and Remazol Brilliant Blue R	[108]
Thermus sp. 2.9	Xylidine, RBBR, Gentian Violet, Methyl Orange	[109]
Geobacillus sp. JS12	Congo red, Malachite green	[110]
Anoxybacillus ayderensis SK3-4	Direct blue 6, acid black 1, direct green 6, direct black 19, and acid blue 93	[111]
Thermosediminibacter oceani	Malachite green (MG) and Congo red	[112]

In the genome of *T. thermophilus* HB27, a laccase that is able to oxidize six different dyes (dye orange, acid red dye, green dye, naphthol brilliant blue, Remazol brilliant blue, Congo red), has been characterized; the enzyme requires an electron shuffle, that supports this reaction for some dyes [107]. Also *Geobacillus* sp. JS12 contains a laccase, LacG, that can decolor these artificial compounds at 70 °C [110]. In the alkaliphilic and thermophilic bacterium *Anoxybacillus* sp. strain UARK-01, the UARK 01 laccase can oxidize the Congo red substrate, one of the most toxic dyes [105]. In addition to the laccases, these extremophiles also have a striking azoreductase, active on different dyes; for example, *Anoxibacillus* sp. PDR2 acts towards the direct black G [113]; moreover, the

degradation of the same dye can be obtained by a thermophilic microflora, consisting of facultative aerobic (*Anoxybacillus flavithermus* strain 52-1A, *Tepidiphilus thermophilus* strain JHK30, *Tepidiphilus succinatimandens* strain 4BON, *Brevibacillus aydinogluensis* strain PDF25, *Bacillus thermoamylovorans* strain DKP and *Geobacillus thermoleovorans* strain NP1) and exclusively anaerobic bacteria (*Thermoanaerobacterium thermosaccharolyticum* strain DSM 571, *Thermoanaerobacterium thermostercoris* strain Buff, and *Caloramator proteoclasticus* strain Uruguayensis) [114]. These examples give a generic view on the potential application of thermophilic oxidoreductases in biological detoxification.

4. Lignocellulosic Biomasses

In the last decades, there is a growing interest in the use of microbes in industrial processing to break waste food and lignocellulose biomasses to produce biofuels and bioproducts. Among renewable resources, non-food lignocellulosic waste biomasses are currently considered among the most promising materials, since they are present in large quantities and at low cost [115]. Every year, a significant amount of lignocellulosic residues is generated worldwide from agricultural wastes, food industry, household garbage, non-food seeds, etc. (see Table 4), causing an increase in environmental pollution. Lignocellulosic wastes are also often improperly stored and recalcitrant to different disposal treatments; moreover, when burnt, they provoke environmental pollution problems. Thus, the reuse and exploitation of such wastes in industrial biotechnology to produce interesting chemicals allows to bypass a part of disposal treatments [116].

Table 4. Percentage content of most common lignocellulosic wastes [117,118].

Lignocellulosic Wastes	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Softwood stems	25–35	25–35	45–50
Hardwood stems	18–25	24–40	40–55
Miscellaneous Corn stover	19	22	39
Wheat straw	15	50	30
Rice straw	18	24	33
Nutshells	30–40	25–30	25–30
Peels	14–20	11	4
Shells	26–30	20–25	40–45
Sorted refuse	20	20	60
Swine waste	n/a	28	6
Solid cattle manure	2.7–5.7	1.4–3.3	1.6–4.7
Grass	10–25	35–50	30–40
Cotton seed hairs	0	5–20	80–95
Leaves	0	80–85	15–20
Sawdust	14–34	71–89	31–64
Paper	0–15	0	85–99
Newspaper	14–19	25–40	40–55
Wastepaper from chemical pulps	5–10	10–20	60–70
Primary wastewater solids	24–29	n/a	8–15

Lignocellulose is a significant component of plant biomass and it consists of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are polymers of different sugars; in particular, the principal constituent of lignocellulosic biomass is cellulose, a polysaccharide composed of β-1,4-linked D-glucose units, widely employed for paper and cardboard production. Instead, hemicellulose is a complex branched polysaccharide formed by a mixture of xylans, mannans, β-glucans, and xyloglucans, depending on the type of wood. In softwood, hemicellulose mainly consists of galactoglucomannan, composed of β-1,4-linked D-glucose and D-galactose units. In contrast, xylan is the constituent of hemicellulose in hardwood, and it is formed of β-1,4-linked D-xylose units, which can be substituted with other monosaccharides [119]. Hemicellulose is closely associated with cellulose filaments and covalently attached to lignin, forming a matrix.

Lignin is an aromatic heteropolymer composed of ether and C–C bonds that link phenylpropanoid aryl-C3 units. The percentage composition of these polymers in waste lignocellulosic biomasses varies as shown in the table below; the content of cellulose, hemicellulose and lignin also changes in a single plant depending on the age, stage of growth, and other conditions [120].

A high number of microorganisms, belonging to both bacterial and archaeal kingdom, possesses complex metabolic pathways able to decompose lignocellulose. Their enzymes can be utilized as biocatalysts for green approaches in several industrial fields, such as paper and pulp industry, food processing and textile sector, agriculture, animal food production, etc. (Figure 4). Furthermore, several microbial based technologies for the exploitation of lignocellulosic wastes as raw materials for producing bioproducts and biofuels were set up. In the specific case of bioethanol production, from early 70 to 2000s, a "first-generation technology" (1G) was developed, in which the biorefinery systems were based on the use of starch/sugar crops (sugar beet, maize, and sugar). However, 1G has several unsustainability issues bound to the great request of crops subtracted to the food chain and the cultivation of large areas (destined for this purpose) that causes deforestation and decrease of biodiversity [121]. As an alternative, in the "second-generation" technologies (2G), lignocellulosic materials are employed as feedstocks; in this respect, lignocellulose is cheap and immediately available in a large amount [122], but the development of tailored technologies is necessary to exploit more recalcitrant components. For example, both 1G and 2G technologies have to simultaneously maximize production yield and reduce costs and environmental impact; in both cases, exploitation of microbial mechanisms and biocatalysis supports the process. The bioethanol production process consists of different phases: biomass pre-treatment, saccharification, fermentation, and distillation. The main difference between the two technologies lies in the complexity of the starting raw material; in the first-generation technologies after pre-treatment of sugarcane and maize, a chemically homogenous material (sucrose and starch) can be easily broken into sugar units by a limited number of enzymes like amylases, amylopullulanases or glucosidases [123]. For the improvement of 1G processes, investigation on α-amylases, α-D-glucosidases, pullulanases and amylopullulanases of thermophilic bacteria and archaea have been carried out. For example, *T. thermophilus* HB27, *Thermoanaerobacter ethanolicus* 39E, *Geobacillus thermoleovorans* NP33, *Rhodothermus marinus*, *Clostridium thermosulfurogenes*, *Clostridium thermocellum*, *Desulfurococcus mucosus*, *Fervidobacterium pennavorans*, *Bacillus stearothermophilus*, *Thermotoga maritima* and some species of the genera *Pyrococcus*, *Thermoanaerobacter*, and *Thermococcus* have been studied since they are able to produce starch degrading enzymes. However, some of their biocatalysts have limited activity with high starch concentration (>30%). Therefore, mesophilic hosts are still the preferred ones for bioethanol production [124,125]. In fact, standardized methodologies for saccharification and fermentation have been mainly optimized in engineered mesophilic yeasts or microbes (*Saccharomyces* and *Zymomonas* spp.) with a high bioethanol production yield [126].

On the other hand, the degradation of lignocellulose is more complicated because the starting matrix is heterogeneous. The lignin removal step requires a significant amount of energy (acid hydrolysis or steam explosion) to release sugar polymers for the subsequent saccharification step [123]. Moreover, in 2G, either in saccharification and fermentation, additional steps are necessary to achieve the complete production of bioethanol from cellulose and hemicellulose; therefore, despite its cheapness and availability, lignocellulosic material implicates a more elaborate treatment process.

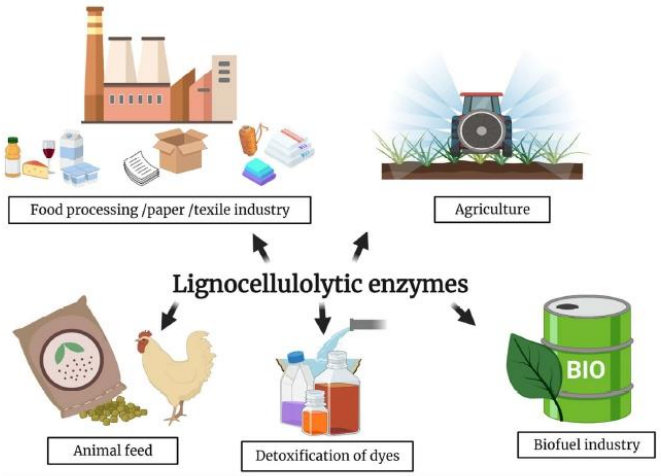

Figure 4. Industrial applications of lignocellulolytic enzymes. In food processes cellulases and xylanases are employed to improve the shelf life of dairy products and to hydrolyze monosaccharides in milk processing; they are also used in winery industries and to decrease viscosity of fruit juice. Also, laccases are used to increase quality of beverages and food, for example eliminating toxic substances. In pulp/paper industry laccases and xylanases enhance pulp bleaching for paper manufacturing, and cellulases improve flexibility and softness of fibers. Lignocellulolytic enzymes can be employed for improving nutrient digestibility of animal feeds, for enhancing color and surface brightness of fabric in textile industry, for textile dye bleaching, for synthesis of complex polymers. In agriculture, they are involved in fruit ripening and defense mechanisms against insects. Laccases are employed in wastewater treatment of colored waters. Lignocellulolytic enzymes are used in biorefinery systems to produce biofuels [103,127,128] (Created with BioRender.com (accessed on 20 January 2021)).

In this context, the use of thermophiles in lignocellulosic biomass degradation has the advantage that higher temperatures and organic solvents can be used, reducing either risks of microbial contamination or energy consumption (because the cooling steps are not necessary), and increasing rates of hydrolysis and product yields [129]. Another attractive progress on the use of renewable lignocellulosic biomass to produce bioethanol or feedstock chemicals consists in setting up microbial based bioprocesses that exploit the synergistic degradative capabilities of thermophilic microorganisms or consortia [130].

4.1. Lignin Degrading Thermozymes

Enzymes that depolymerize lignin are isolated principally from the white-rote fungi; some of these enzymes are manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP), and laccase. In particular, laccases are a heterogeneous subfamily of multicopper oxidases (MCOs) that can be involved in several biological processes like lignolysis and detoxification of dyes (see above) [131].

Recent studies have described very promising thermostable laccases able to degrade lignin derived from thermophilic microorganisms such as *Bacillus* sp. PC-3 and sp. FNT with optimum activity temperatures of 60 and 70 °C, respectively [132,133]. The first archaeal laccase was characterized from the halotolerant *Haloferax volcanii*, a promising microorganism for the lignin break-down purposes; this archaeon grows up to 50 °C and it possesses a stable glycoprotein, the laccase LccA that acts on several substrates at elevated temperature (55 °C), high salt concentrations (0.1 to 1.4 M) and it can maintain its activity also in organic solvents [134]. Also, many *Thermus* species have laccases that can be employed in lignin degradation; laccase of *Thermus* sp. 2.9 can retain 80% of its activity at 70 °C for 16 h and is able to successfully delignify *Eucalyptus* biomass [135].

Two strains of *T. thermophilus* (HJ6 and HB27) produce laccases capable of maintaining an optimal temperature range of activity at 85–90 °C for reactions up to 1 h [107,136]. Moreover, two hyperthermophilic bacteria present laccases with remarkable heat stability; a chemolithoautotrophic bacterium, *Aquifex aeolicus*, expresses a multicopper oxidase with an optimal temperature of 75 °C but that preserves its activity at 80 and 90 °C for up to 9 and 5 h, respectively [137]. Furthermore, the laccase-like multi-copper oxidase of *Thermobaculum terrenum* is extremely thermostable with a half-time of inactivation of 2.24 days at 70 °C and 350 min at 80 °C and pH 7 [138].

These features suggest that an impressive compromise between thermostability and lasting activity exists for these enzymes; for this reason, they are considered as promising tools to degrade the lignin component of biomasses.

4.2. Cellulose and Hemicellulose Degrading Thermozymes

Several thermophilic archaea and bacteria are able to produce a considerable amount of promising cellulose/hemicellulose degrading enzymes. These thermophilic enzymes, differently from their mesophilic counterparts, have the advantage that they can be added immediately after the thermochemical pre-treatment of biomass, making the cooling steps not necessary, increasing conversion efficiency, and saving time [139].

Whereas cellulose can be completely depolymerized through endoglucanases, exoglucanases and β-D-glucosidases, the total degradation of hemicellulose requires a wider pool of enzymatic activities (i.e., β-xylosidases, β-xylanases, β-glucuronidases, *β*-mannanase, *β*-mannosidase, *α*-galactosidase, etc.).

Several thermophilic cellulose degrading bacteria have been isolated from distinct environments like hot springs, compost systems and soil. They include different species belonging to the genera *Actinomadura, Alicyclobacillus, Anoxybacillus, Acidothermus, Bacillus, Caldanaerovirga, Caldicellulosirupto, Cellulomonas, Clostridium, Dictyoglomus, Geobacillus, Paenibacillus, Nesterenkonia, Paenibacillus, Pyrococcus, Rhodothermus, Sulfolobus, Thermoanaerobacterium,* and *Thermotoga*; they can produce both cellulose and hemicellulose degrading enzymes that can raise the rates of biomass hydrolysis if they are used in industrial bioprocesses [140].

Some examples of remarkable thermophiles include the following ones.

Acidothermus cellulolyticus 11B, isolated from a hot spring in Yellowstone National Park, produces a tri-functional enzyme that can break down birchwood xylan with high efficiency; in fact, this enzyme has endo-xylanase, arabinofuranosidase, and acetyl-xylan esterase activities [141].

Two thermoalkaline species of *Anoxybacillus (kamchatkensis* NASTPD13 and sp. 3M) express many xylanases and β-xylosidases, respectively, highly resistant to alkaline and acidic pHs, denaturing agents and organic solvents [142,143]. Also the facultative anaerobic *Bacillus coagulans* MA-13, which lives at an optimal temperature of 55 °C, secretes an endo-1,4-β-glucanase which can act from 37 to 60 °C. *B. coagulans* MA-13 is also able to ferment sugars derived from pre-treatment of lignocellulose to lactic acid in the presence of inhibitors; in fact, it was proved that this bacterium can grow and ferment in bioreactors containing 95% hydrolysate [144]. At the same time, seed culture pre-adaptation of *B. coagulans* MA-13, before simultaneous saccharification and fermentation step, can improve the production of lactic acid; again, it has a pool of interesting intra- and extracellular enzymes with glycosyl hydrolyzing activities that make *B. coagulans* MA-13 useful for increasing nutritional value of food [145,146].

Clostridium thermocellum has a non-enzymatic scaffolding protein bound with different enzymatic subunits that simultaneously degrade cellulose and hemicellulose [147]. In 2018 a new cellulolytic strain was identified in the *Chryseobacterium* genus, which produces an enzyme with a double cellulase/xylanase activity working either on carboxymethylcellulose and birchwood xylan [148].

Instead, the anaerobic *Caldicoprobacter* sp. CL-2, isolated from bovine manure compost, has a xylanase activity showing a modular structure with a glycoside hydrolase

domain coupled with a carbohydrate binding module [149]. Another hyperthermophilic microorganism from geothermal springs that produce miscellaneous glycoside hydrolases is *T. maritima*; it has an endoglucanase enzyme (Tm_Cel5A) with an optimum T of 80 °C and pH of 4.8. Tm_Cel5A is a peculiar GH5 (glycoside hydrolase family 5) enzyme with an unusual activity because it can act both on glucan and mannan based polysaccharides, while the other GH5 hydrolysing enzymes degrade either cellulose or mannans [150].

Dictyoglomus turgidum is another thermophilic microorganism that displays a set of genes encoding putative enzymes with glycosyl hydrolyse activity; this not yet well characterized thermophile has an endo-1,4-β-mannanase, *Dtur*CelB, with a high thermoresistance (Tm of 88 °C) and a good thermal and pH stability; it is also resistant to chemicals and has been analyzed in an enzymatic cocktail able to cut-off cellulose and hemicellulose [151,152]. In fact, different thermophilic biocatalysts can be utilized synergistically for the complete breakdown of hemicellulose sugars (pentose and hexose) of lignocellulosic material. The two recombinant thermophilic enzymes, the above mentioned *Dtur*CelB from *D. turgidum* and the α-galactosidase from *T. thermophilus*, can be tested in the lignocellulose pre-hydrolyzing step right before the saccharification step [153].

Other two thermophiles, *Thermotoga neapolitana* 5068 and *T. thermophilus*, display the most thermoactive (~100 °C) and thermostable (half-life of 30 h at 70 °C) α-galactosidase activities, respectively [154,155].

Archaeal glycoside hydrolyzing enzymes have also been exploited to improve biomass degradation processes. For example, the hyperthermophilic archaeon *Sulfolobus shibatae* encodes an endo-1,4-β-D-glucanase that accomplishes the break-down of carboxymethylcellulose, xylan and barley β-glucan [156]. *Pyrococcus furiosus* produces extracellular endoglucanases, intracellular glucosidases, and different intra- and extracellular amylases with a high thermostability in the range 80–100 °C; one of these enzymes, a β-glycosidase, is immobilized and used in industrial process of lactulose production [157]. Furthermore, *Saccharolobus solfataricus* expresses a membrane-bound xylanase, an extracellular endoglucanase, intra- and extracellular galactosidases, an extracellular xylosidase and an intracellular mannosidase. In the case of *S. solfataricus*, its thermozymes have been used as model for engineering mesophilic enzymes in order to improve their thermostability; for instance, the β-glycosidase of this archaeon, that shows a maximal activity above 95 °C, represents a fine example of an efficacious heterologous production in a yeast expression system [158].

In recent years, several studies are focused on taking advantage on thermophilic communities that can provide a high hydrolyzation rate of lignocellulosic material (Table 5). For example, consortia formed by bacterial and fungal microorganisms such as Alcaligenaceae, Burkholderiacea, *Thermoamylovorans*, Xanthomonadaceae, *Mycobacterium*, *Talaromyces* and *Rubrobacter* can decompose biomasses with a high content of lignin [159].

Furthermore, high throughput genome sequencing, transcriptomics, proteomics, metagenomic, and other omics techniques together with metabolic engineering strategies and bioinformatic tools have contributed significantly to explore a considerable amount of novel thermophilic lignocellulolytic microorganisms and enzymes.

Table 5. Examples of thermophilic bacteria and archaea able to hydrolyze the lignocellulose.

Lignocellulosic Component	Microorganism	Temperature	Ref.
Lignin	*A. aeolicus*	89 °C	[137]
	Bacillus sp. PC-3	55–92 °C	[132]
	Bacillus sp. FNT	50–55 °C	[133]
	H. volcanii	50 °C	[160]
	Thermus sp. 2.9	65 °C	[135]
	T. thermophilus HJ6	80 °C	[136]
	T. thermophilus HB27	70 °C	[107]
	T. terrenum	67 °C	[138]
	Fungal and bacterial consortium	55 °C	[159]

Table 5. *Cont.*

Lignocellulosic Component	Microorganism	Temperature	Ref.
Cellulose and hemicellulose	*A. cellulolyticus* 11B	70 °C	[141]
	A. kamchatkensis NASTPD13	60 °C	[142]
	Anoxybacillus sp. 3M	55 °C	[143]
	B. coagulans MA-13	55 °C	[144]
	Brevibacillus borstelensis SDM	50 °C	[161]
	C. thermocellum	60 °C	[147]
	Chryseobacterium sp.	55 °C	[148]
	Caldicoprobacter sp. CL-2	60–75 °C	[149]
	D. turgidum	75–80 °C	[151]
	T. maritima	80 °C	[150]
	T. neapolitana 5068	70–80 °C	[162]
	T. thermophilus HB27	70 °C	[107]
	P. furiosus	100 °C	[158]
	S. shibatae	80 °C	[156]
	S. solfataricus	80 °C	[158]

The development of genome editing tools also represents a new approach to address biomass degradation by microorganisms; in fact in a next future, the genome manipulation of thermophilic bacteria will make possible to develop fine bioprocessing microbial strains, that will be capable of better performing degradation of lignocellulose [163]. Thus, thermophiles have a great potential to be considered as a suitable platform for metabolic engineering to produce various biomolecules and/or valuable chemicals from lignocellulosic biomasses.

5. Engineering of Thermophiles

Thermophilic microorganisms have unique biochemical and physiological characteristics with important biotechnological implications. Thermophilic microorganisms can be used in numerous applications, such as biocatalysis, or as sources of thermoactive or thermostable enzymes. However, unfortunately, their employment as *whole-cell* systems is limited by the lack of easily usable genetic systems. This situation has changed recently, with unprecedented progress in genetic tools for extremophilic microorganisms, and the use of these microorganisms as platforms has become possible.

Significant studies have been made to develop and improve molecular genetic techniques for thermophilic microorganisms in the past decade, either belonging to the bacterial or archaeal kingdom. A significant challenge for genetic modification in thermophiles is the choice of a selectable marker to screen positive transformants. The antibiotics typically used in mesophiles often target cell components specific to bacteria and are ineffective against the archaeal species. Even in cases where antibiotics are useful, both the antimicrobial compound and the gene product that confers resistance must be stable at elevated temperatures. Due to the low efficiency of the heat-resistant antibiotic selection markers, usually nutritional selection systems such as enzymes essential for the synthesis of amino acids can be used. To date, genetic techniques have been obtained for ten such archaea, including *Metallosphaera*, *Sulfolobus*, *Thermococcus*, and *Pyrococcus* species [164–167].

The creation of genome editing tools enabling stable integration of genetic elements into host chromosomes is crucial for industrial applications, where plasmid instability becomes problematic and volumes of antibiotics on an industrial scale are very polluting. In principle, two alternative approaches for developing genome editing tools can be adopted for thermophilic bacteria; one is by adapting mesophilic protocols to function at elevated temperatures. The second is to seek alternative means of genome editing from thermophilic springs. Several examples describe the use of homologous recombination to knock out or replace chromosomal genes in thermophilic bacteria. In 2012, Suzuki and co-workers developed a *pyr*F/*pyr*R counterselection system for *Geobacillus kaustophilus*, enabling marker-free genome editing at 60 °C [168].

Another widely used system to obtain genetic manipulation of thermophilic bacteria is the *Cre/loxP* site-specific recombination [169]. This recombination is performed between two *loxP* sites using a Cre recombinase, *loxP* is a 34 bp consensus DNA sequence with a central spacing region of 8 bp, which defines its orientation, flanked by two 13 bp palindromic sequences, which are the Cre binding sites. The *Cre/lox* system's effectiveness in a broad spectrum of biological species and a wide variety of applications has made this technology indispensable for in vivo genetic manipulation. This system allows various recombination types, such as conditional recombination, intermolecular recombination and time and space specific recombination [169]. Recently, a *Cre/lox* system was developed for the thermophilic bacterium *T. thermophilus* HB27 [170], leading to the development of a highly efficient method of destroying multiple genes to facilitate genetic manipulation of this bacterium. The most important advantage that made easier to develop genetic tools for *T. thermophilus* is the constitutive expression of a natural competence system in several strains [171]. Several plasmids have been developed to transform *T. thermophilus*, and some of these, suitably modified using regions of homology to the chromosome, have been used to stimulate homologous recombination, obtain deletions of genes, thus allowing the study of the in vivo function of specific proteins [172].

For these reasons, *T. thermophilus* is considered a biological model for functional studies and a right candidate for biotechnological applications. However, its efficient defense system against the exogenous DNA can be an impairment since it can destroy the cloning vectors used for transformation; in 2014, Daan C. Swarts and co-workers identified *Tt*Ago, a protein belonging to the Argonaute family as the protein responsible for the prevention of the uptake and propagation of foreign DNA [173]. The researchers observed that the protein generally attacks the AT-rich regions of double-stranded DNA, leading to the complete plasmid degradation by other nucleases [173].

The Rise of the CRISPR-Cas Era

Until 2013, the principal genome editing tools were the zinc finger nucleases, the transcription activator-like effector nucleases and intrinsic homologous recombination systems [174–176]. These systems use artificial fusion proteins composed by an engineered DNA-binding domain fused to the non-specific nuclease domain of the restriction enzyme FokI. These systems were extensively used for the genome editing of eukaryotic microorganisms. A new technology for genome editing rose based on RNA-guided engineered nucleases (CRISPR-Cas9 system) in the last decades. Although the CRISPR array was discovered in the late 1980s [177], its function remained unknown until 2005 [178]. Only in 2007, it was concluded that it represented a bacterial innate immunity system [179,180]. The transition of the CRISPR/Cas system from a biological phenomenon to a tool for genome engineering occurred when it was shown that the target DNA sequence could be reprogrammed simply by changing 20 nucleotides in the crisprRNA (crRNA) and that the targeting specificity of the crRNA could be combined with the structural properties of the tracrRNA (trans-activating crisprRNA) in a chimeric single guide RNA (sgRNA) [181] (Figure 5).

Furthermore, the evidence that sgRNAs with different specificities could be produced made it possible to modify more loci simultaneously, giving a connection to the so-called CRISPR-mania [182]. The various genome editing applications pioneered in human and animal cells have recently been transferred back to bacteria to carry out genome editing and transcriptional control, as well as genome-wide screens. In fact, the CRISPR-Cas system was used to obtain some genetically modified prokaryotes. However, one problem for applying this genetic editing tool to thermophilic microorganisms, is that it is based on a mesophilic system. In recent years the research has been going towards the search for Cas proteins from thermophiles; in fact, a thermostable genome editing tool was developed based on a thermophilic Cas9, that can be used up to 55 °C and contains everything necessary for genome editing in a single plasmid; with the advent of the ThermoCas9,

genome manipulation in moderate thermophilic bacteria becomes possible, making the editing process much more comfortable and less time-consuming [183].

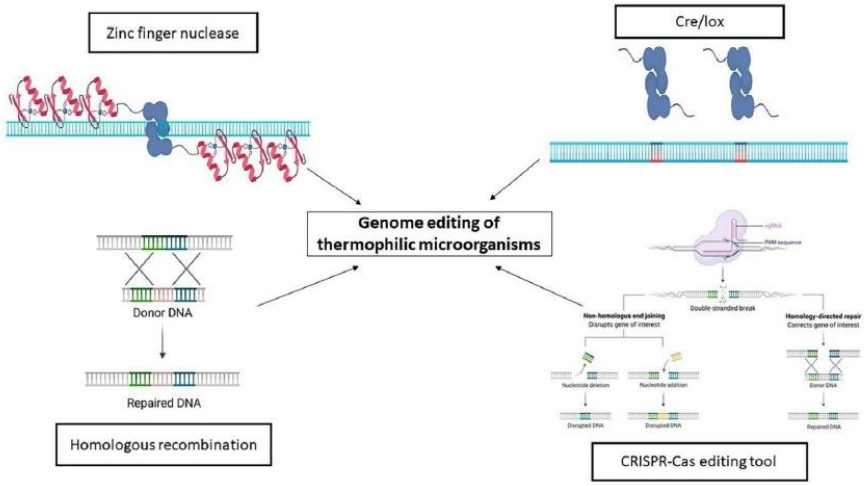

Figure 5. Schematic representation of the genome editing tools for thermophilic microorganisms. The Zinc finger nuclease is the first genome editing tool setup for thermophilic microorganisms. The most common is the spontaneous homologous recombination. In the last decades, the Cre/lox and CRISPR-Cas based tools were adapted for thermophiles (Created with BioRender.com (accessed on 20 January 2021)).

A genome-editing tool was recently developed for moderate thermophilic bacteria obtained using the Cas12a from *Francisella novicida* [184]; this system allowed to obtain knockout mutants in less than one week with high editing efficiencies. *Fn*Cas12a has an interesting potential for the genome editing of many thermophilic bacteria and archaea.

Cas9 and Cas12a are multidomain CRISPR-associated nucleases that can cleave complementary DNA targets using a guide RNA. The Cas9 belongs to type II-a, while the Cas12a to type V-a. The first enzyme is the best characterized and utilizes nuclease for genome-editing purposes. In the last years, Cas12a has emerged as a potential alternative. These two enzymes have distinct evolutionary origins and present different structural architectures, resulting in specific molecular mechanisms; in fact, the nuclease activities of Cas9 and Cas12a and the resulting DNA repair outcomes are affected by circumstantial factors such as cell type, target sequence, and genomic context [185]. Their biological differences influence their application as genome editing tools: in some cases, the Cas9 activity is more suitable for some organisms, in other cases the best option is to use Cas12a. Instead, Cas9 and Cas12a and their engineered variants are highly complementary in their properties and together build up a powerful and versatile toolkit.

6. Conclusions

Extremophiles represent a class of microorganisms very interesting for their ability to live in harsh conditions, not only high temperature, but also extreme pHs and high salinity concentrations. These peculiar characteristics make them and their biocatalysts very promising tools for industrial and environmental applications. Thermophilic extremophiles stand out in biometallurgy for biomonitoring and bioremediation, as well as in degradation of organic biomasses to transform them into resources ready to be re-used. In fact, in

addition to the biofuel production, hydrolyzing extremozymes have a wide range of applications in the food, feed, beverage, textile, pulp and paper industry.

Improved knowledge in *omic-era* and the increasing need to address environmental pollution with green processes drive biotechnological research in search of microorganisms that can replace chemical processes. The newly available thermophilic genome editing tools based on the CRISPR-Cas system, open the way for the complete achievement of these goals in various industrial fields. In fact, the rise of the "CRISPR-Cas *era*" makes possible the application of engineered extremophiles as a *whole-cell* platform.

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Chapter [6]: General Conclusions

Environmental pollution is one of the most serious problem of the modern age; among heavy metals, arsenic represents a harsh pollutant able to contaminate air, water and soil, and also very harmful for human health. Most of the current systems for monitoring and restoring the environment require several expensive and hard instrumentations; green biotechnologies drives to the employment of easier and possibly cheaper processes and devices [1,2].

In this context, the exploitation of thermophiles and their components to face the environmental pollution caused by heavy metals is an increasing strategy for different applications. The choice of thermophiles is very advantageous, in comparison to their mesophilic counterpart, because many of them are highly resistant to toxic metals and other stress conditions; this is due to their adaptation to survive at high concentrations of heavy metals present in some extreme environments, as particular hydrothermal vent, or polluted sites.

Therefore, starting from the study of the heavy metal resistance mechanisms in thermophiles, it is possible to apply such knowledge to address many biotechnological issues: metal bioremediation, biomining and development of metal biosensors [3].

This PhD project has been structured along two thematic areas, both based on this topic: the development of biosensors for the monitoring of toxic metals using a thermostable arsenate reductase and the characterization of new extremophilic microorganisms in order to identify novel resistance mechanisms as basis to set up bioremediation processes. Heavy metals, and in particular arsenic, have been the main target of this research.

In particular, the arsenate reductase from *Thermus thermophilus*, *Tt*ArsC [4], has been considered as the biorecognition component of biosensors for the arsenic detection, since it had been already exploited for this application [5,6] and we knew its potentialities. In particular, the biosensor previously developed consisted in the conjugation of *Tt*ArsC on gold nanoparticles through polyethylene glycol immobilization, and optical detection. In this thesis, we developed an electrochemical biosensor based on chimeric proteins (ArsC-Vmh2 and Vmh2-ArsC) endowed with both the arsenic sensing ability of *Tt*ArsC and the adhesive properties of Vmh2 [7], a self-assembling amyloid protein, in order to have a one-step easy immobilization of the biomaterial on the support. From the design point of view, Vmh2 had been previously exploited as scaffold for the construction of chimeric proteins, but this was the first time that Vmh2 was expressed at the N-terminal of a

chimera. This location has positive effect on folding, yield of production and renaturation of Vmh2-ArsC, in comparison to ArsC-Vmh2. Furthermore, also the catalytic activity of Vmh2-ArsC is slightly higher than that of ArsC-Vmh2 when the proteins are immobilized on hydrophobic supports, suggesting that the expression of Vmh2 at the N-terminal of a chimeric protein also favours the adhesion on surfaces, improving the catalytic activity in terms of substrate accessibility. The chimeras were immobilized on the surface of gold electrodes, and thanks to the interaction of *Tt*ArsC with As(III), this latter was successfully sequestered from solutions and electro-reduced at acidic pHs, through Square Wave Voltammetry (SWV). Despite the binding constants (650 ± 100 L mol^{-1} for ArsC-Vmh2 and 1200 ± 300 L mol^{-1} for Vmh2-ArsC) need to be increased in order to possibly use these bioelectrodes for As(III) biosensing, to the best of our knowledge, this is the first example of the use of a thermostable arsenate reductase in an enzyme-based electrochemical arsenic biosensor and opens the way for improvements to be achieved either by further enzyme engineering strategies or by the use of advanced nanostructured electrodes [8].

Using another approach, we investigated the phosphatase activity of *Tt*ArsC. This secondary activity does not require the involvement of the complex system Trx-TR and can be easily followed with a colorimetric substrate (pNPP). Therefore, investigations on the effect of As(V) as inhibitor of the phosphatase activity revealed the possibility to employ *Tt*ArsC as an As(V) optical biosensor. In particular, despite both As(V) and As(III) behave as non-competitive inhibitors, the affinity towards the first is about 25 times higher, probably because As(V) is the natural substrate of the arsenate reductase. As fact, it is possible to see the complete inhibition of the reaction with naked eye from 50 µM of As(V) onwards (with the gold nanoparticles biosensor the reaction was visible with naked eye at 85 µM [5,6]), and using a spectrophotometer the system exhibit a sensitivity of 0.53 ± 0.03 mU/mg/µM and a LOD of 0.28 ± 0.02 µM. Moreover, while this complete inhibition is achieved within a micromolar range of As(V) concentration, interestingly, higher concentrations (in millimolar ranges) are required with As(III) and almost all the other heavy metals to obtain comparable inhibition levels. Therefore, since the specificity of this system is remarkable, and its use is very simple, it is possible to foresee future applications in the arsenic biosensors field.

Concerning the second thematic area of this PhD thesis, focused on the characterization of new thermophilic heavy-metal resistant microorganisms to exploit in potential bioprocesses, we performed

sampling in a hot mud pool in the hydrothermal volcanic area of Pisciarelli, near Naples in Italy. This acidic sulfate area located close to the famous Solfatara crater is an arsenic-rich area, characterized by intense endogenous diffuse and fumarolic water-dominant outgassing activity. We succeeded in isolating a microorganism growing optimally at a temperature of 60 °C and pH 7 identified as a *G. stearothermophilus* by ribotyping, 16S rRNA sequencing and mass spectrometry analyses, that we named *G. stearothermophilus* GF16 [9]. In a subsequent sampling, we isolated a new microorganism with an optimal growth temperature of 55 °C at pH 4, identified as a new strain of *Alicyclobacillus mali* by genomic analyses, that we called *A. mali* FL18 [10]. Our experiments demonstrated that both microorganisms can be considered arsenic resistant in agreement with the natural environmental setting composition as well. Moreover, genomic insights of *A. mali* FL18 revealed the putative molecular determinants of its arsenic resistance system, as well as to putative genes responsible for resistance to other heavy metals and antibiotics.

Microbes flourishing in primordial niches rich of such toxic metalloids have been subjected to natural selection for a long time. Both these studies highlight the striking adaptation capabilities of these thermophiles and their tolerance to extreme conditions, making them promising candidates for further investigations either to address bioremediation of heavy-metal pollution and also to identify new thermostable proteins/enzymes for industrial applications. In particular, with the set-up of genome editing tools based on thermophilic CRISPR-Cas systems, a revolution in the biotechnological application of thermophiles in industrial fields is expected [11].

In conclusion, the results obtained during this PhD project consolidate the principles at the basis of the employment of thermophilic microorganisms and enzymes in the development of biosensors and bioprocesses for environmental biotechnologies.

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10. Aulitto, M.; Gallo, G.; Puopolo, R.; Mormone, A.; Limauro, D.; Contursi, P.; Piochi, M.; Bartolucci, S.; Fiorentino, G. Genomic Insight of Alicyclobacillus mali FL18 Isolated From an Arsenic-Rich Hot Spring. *Frontiers in Microbiology* **2021**, *12*.
11. Gallo, G.; Puopolo, R.; Carbonaro, M.; Maresca, E.; Fiorentino, G. Extremophiles, a Nifty Tool to Face Environmental Pollution: From Exploitation of Metabolism to Genome Engineering. **2021**.

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Appendix I

List of publications

- Gallo G, **Puopolo R**, Carbonaro M, Maresca E, Fiorentino G. Extremophiles, a Nifty Tool to Face Environmental Pollution: From Exploitation of Metabolism to Genome Engineering
  2021, Int J Environ Res Public Health, Vol. 18:5228
  DOI= 10.3390/ijerph18105228
- Aulitto M, Gallo G, **Puopolo R**, Mormone A, Limauro D, Contursi P, Piochi M, Bartolucci S, Fiorentino G.
  Genomic Insight of *Alicyclobacillus mali* FL18 Isolated From an Arsenic-Rich Hot Spring.
  2021, Front Microbiol, Vol. 12:669
  DOI=10.3389/fmicb.2021.639697
- **Puopolo R**, Sorrentino I, Gallo G, Piscitelli A, Giardina P, Le Goff A, Fiorentino G.
  Self-assembling thermostable chimeras as new platform for arsenic biosensing.
  2021, Sci Rep, Vol. 11: 2991.
  DOI=10.1038/s41598-021-82648-9
- **Puopolo R**, Gallo G, Mormone A, Limauro D, Contursi P, Piochi M, Bartolucci S, Fiorentino G.
  Identification of a New Heavy-Metal-Resistant Strain of *Geobacillus stearothermophilus* Isolated from a Hydrothermally Active Volcanic Area in Southern Italy.
  2020, Int J Environ Res Public Health, Vol. 17: 2678.
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- Gallo G, **Puopolo R**, Limauro D, Bartolucci S, Fiorentino G.
  Metal-Tolerant Thermophiles: From the Analysis of Resistance Mechanisms to their Biotechnological Exploitation.
  2018, Open Biochem J, Vol. 12: 149-160.
  DOI=10.2174/1874091X01812010149

Author's contribution

- Extremophiles, a Nifty Tool to Face Environmental Pollution: From Exploitation of Metabolism to Genome Engineering Conceptualization, G.G., **R.P.**, M.C., E.M., and G.F.; investigation, G.G., **R.P.**, M.C., and E.M.; data curation, G.G., **R.P.**, M.C., and E.M.; figure preparation, **R.P.** and M.C.;

writing—original draft preparation, G.G., **R.P.**, M.C., E.M., and G.F.; writing—review and editing, G.G., **R.P.**, M.C., E.M., and G.F.; visualization, G.G., **R.P.**, M.C., E.M., and G.F.; supervision, G.F.; project administration, G.F.; funding acquisition, G.F. All authors have read and agreed to the published version of the manuscript.

- Genomic Insight of *Alicyclobacillus mali* FL18 Isolated From an Arsenic-Rich Hot Spring.
  M.A., G.G. and **R.P.** have contributed equally to this work and share first authorship. M.A., G.G., and **R.P.**: data curation. M.A., G.G., **R.P.**, M.P., A.M., S.B., and G.F.: investigation. M.A., **R.P.**, G.G., M.P., A.M., and GF: methodology. D.L., P.C., M.P., S.B., and G.F.: supervision. M.A., G.G., **R.P.**, M.P., A.M., S.B., and G.F.: writing—review and editing. All authors have read and agree to the published version of the manuscript.
- Self-assembling thermostable chimeras as new platform for arsenic biosensing.
  G.F. and A.LG. designed experiments, **R.P.** and I.S. performed experiments, G.G. performed bioinformatic analyses, **R.P.**, A.LG. and G.F. wrote the manuscript, **R.P.** and A.LG. prepared graphical items, P.G. and A.P. contributed to the interpretation of the results and to the final version of the manuscript. All the authors reviewed the manuscript.
- Identification of a New Heavy-Metal-Resistant Strain of *Geobacillus stearothermophilus* Isolated from a Hydrothermally Active Volcanic Area in Southern Italy.
  Data curation, **R.P.**; investigation, **R.P.**, G.G., M.P., A.M., S.B., and G.F.; methodology, **R.P.**, G.G., M.P., A.M., and G.F.; supervision, D.L., P.C., M.P., S.B., and G.F.; writing—review and editing, M.P. and G.F. All authors have read and agree to the published version of the manuscript.
- Metal-Tolerant Thermophiles: From the Analysis of Resistance Mechanisms to their Biotechnological Exploitation.
  G.G. and **R.P.** investigation and writing; D.L., S.D. and G.F. supervision and review. All authors have read and agree to the published version of the manuscript.

Appendix II – Participation to congresses

List of posters

- **Puopolo R**, Sorrentino I, Gallo I, Piscitelli A, Giardina P, Le Goff A, Fiorentino G.
  Through new platforms for arsenic biosensing: self-assembling arsenate reductase chimeras
  FEBS 2021 - 45th FEBS Congress – July 3-8, 2021 - Ljubljana (Slovenia)
- **Puopolo R**, Contursi P, Piscitelli A, Giardina P, Fiorentino G.
  Self-Immobilizing Arsenate Reductase Chimeras useful for Arsenic Biosensing
  IFIB 2020 - International Forum on Industrial Biotechnology and Bioeconomy – October 1-2, 2020 – Rome (Italy)
- Gallo G, Mougiakos I, Bianco M, **Puopolo R**, Bartolucci S, van der Oost J, Fiorentino G.
  Development of a Whole-Cell Optical Biosensor for the Detection of Arsenic and Cadmium: A Genomic Dna-Based Approach
  IFIB 2020 - International Forum on Industrial Biotechnology and Bioeconomy – October 1-2, 2020 – Rome (Italy)
- Piscitelli A, Stanzione I, **Puopolo R**, Sorrentino I, Fiorentino G, Giardina P.
  Straightforward immobilization of chimeric proteins
  SIB 2019 60th Congress – September 18 – 20, 2019 – Lecce (Italy)
- **Puopolo R**, Piscitelli A, Giardina P, Bartolucci S, Fiorentino G.
  An Arsenate Reductase Hydrophobin Chimera for Arsenic Biosensing
  2° Workshop BIO/10 - Docenti e Ricercatori di Biochimica della Campania - May 17th – Naples (Italy).
- Gallo G, **Puopolo R**, Antonucci I, Limauro D, Bartolucci D, Fiorentino G.
  A new microbial isolate of *Geobacillus stearothermophilus* to address toxic metal pollution.
  Extremophiles 2018 - 12th Edition of the International Congress on Extremophiles - September 16-20, 2018 - Ischia (Naples, Italy).

List of oral presentations

- Abdalrazeq M, Curci N, Liberti D, Mirpoor F, **Puopolo R**, StanzioneI.

"Dis"equalities in science: a mirror of the society. An environmental pollution overview.
Riunione Nazionale "A. Castellani", dei Dottorandi di Ricerca in Discipline Biochimiche – December 18th – Brallo (Padova, Italy).

- **Puopolo R**. Detection of Metals section. FLAshMoB Second-year meeting - 17th July 2020.
- **Puopolo R**, Contursi P, Piscitelli A, Giardina P, Fiorentino G. **Protein chimeras for Arsenic Biosensing -** (Oral presentation) Scifed Group & iNano-2020 - Webinar on Nanotechnology - June 15-17, 2020.

Congresses organization

- **Member of the organising committee** of the II Industrial Biotechnology Congress: **BioID&A** (Biotechnology Identity and Application) held in Naples on October 28th, 2019.
- Organization as **Member of the Department of Biology** (Università degli Studi di Napoli Federico II) of the Scientific Manifestation "Futuro Remoto - Essere 4.0".
  To Be Smart: Microorganisms as protagonists of the Revolution 4.0 – Naples, 21-24/11/2019
- Organization as **Member of the Department of Biology** (Università degli Studi di Napoli Federico II) of the Scientific Manifestation "Futuro Remoto- Ri-Generazioni".
  Regenerating the Environment: Microorganisms as protagonists in Bioremediation and Biorefinery – Naples, 8-11/11/2018

Appendix III

Experience in foreign laboratories

DEPARTEMENT DE CHIMIE MOLECULAIRE

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Grenoble, le 21/04/2021

Confirmation Letter

Rosanna Puopolo worked as a visiting researcher in our laboratory from the 16/09/2019 to 12/03/2020. The subject of her work was the development of chimeras-based biosensors.

Dr Alan Le Goff

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