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Identification of *Chromobacterium violaceum* genes with potential biotechnological application in environmental detoxification

Marta S.P. Carepo[1], Juliana S. Nina de Azevedo[1], Jorge I.R. Porto[2], Alexandra R. Bentes-Sousa[2], Jacqueline da Silva Batista[2], Artur L.C. da Silva[1] and Maria P.C. Schneider[1]

[1]Departamento de Genética/Laboratório de Polimorfismo de DNA, Universidade Federal do Pará, Campus Universitário do Guamá Rua Augusto Correa, 01, 66075-970 Belém, PA, Brasil
[2]Instituto Nacional de Pesquisas da Amazônia/CPBA, Caixa Postal 478, 69011-970 Manaus, AM, Brasil
Corresponding author: M.P.C. Schneider
E-mail: paula@ufpa.br

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ABSTRACT. *Chromobacterium violaceum* is a Gram-negative bacterium found in a wide variety of tropical and subtropical ecosystems. The complete genome sequence of *C. violaceum* ATCC 12472 is now available, and it has considerable biotechnological potential for various applications, such as environmental detoxification, as well as medical and agricultural use. We examined the biotechnological potential of *C. violaceum* for environmental detoxification. Three operons, comprising the *ars* operon, involved in arsenic resistance, the *cyn* operon, involved in cyanate detoxification, and the *hcn* operon, encoding a cyanase, responsible for biogenic production of cyanide, as well as an open reading frame, encoding an acid dehalogenase, were analyzed in detail. Probable catalytic mechanisms for the enzymes were determined, based on amino acid sequence comparisons and on published structural information for these types of proteins.

Key words: *Chromobacterium violaceum*, Environmental detoxification, Biotechnological potential

INTRODUCTION

The sequencing of the genomes of organisms has given a set of gene products that may yield biotechnological products. As recently reviewed, *Chromobacterium violaceum* encodes a certain number of genes with pharmacological, biotechnological and industrial properties (Durán and Menck, 2001). The complete genome sequence of *C. violaceum* has recently been published (Vasconcelos et al., 2003), and as a consequence of finishing the genome of this bacterium, most commonly found in soil and water in the tropics and subtropics, the Brazilian genome network has been applied for the patents of a series of useful genes coding enzymes and proteins. Such genes could potentially be used in the biotechnological and pharmaceutical industries. Among these applications are synthesis of medically relevant compounds, environmental detoxification, gold recovery, agricultural applications as biocontrol agents against insects, fungi and nematodes, synthesis of biodegradable plastic, and cellulose biosynthesis.

Industrial and agricultural utilization of chemicals has led to an increasing accumulation of pollutants in our environment. Organic chemicals and heavy metal contaminations are widespread throughout the world, and they are becoming a serious problem for public health. Technological development has given rise to numerous contamination risks; however, now it is clear that there is no possible development if the associated risks are not minimized. Industrial biotechnological applications of bacteria and plants are simply the harnessing of natural processes for commercial purposes. One traditional example is the utilization of microorganisms to extract metals from ores (Rawlings, 2002). It is well known that bacteria are involved in various types of biogeochemical cycles in nature. Some of these bacteria have the ability to use these metal compounds as electron donors in energy transduction processes. In these oxidation processes bacteria can transform toxic compounds into other less toxic materials, diminishing the risks associated with their disposal in the environment (Mukhopadhyay et al., 2002; Lloyd, 2003; Akcil, 2003).

Taking into account that biodegradation is a highly exploitable biotechnological application of microorganisms and their genes, we sought to identify genes of the *C. violaceum* genome with potential for environmental detoxification. The arsenic resistance operon, the cyanate operon and the acid dehalogenase open reading frame (ORF) were examined. Cyanide biogenesis in *C. violaceum*, associated with gold recovery, was also analyzed.

ARS RESISTANCE OPERON

In nature, arsenic (As) can be found in insoluble forms in combination with sulfur, such as AsS and As_2S_3, or as arsenopyrite (FeAsS), an iron-sulfur compound. The oxidation of these compounds gives rise to chemical forms that are more toxic to human life, such as arsenate (As(V)) and arsenite (As(III)). Arsenate is present in oxide environments, and it binds strongly to sediments. Arsenite can be obtained from arsenate under anaerobic conditions, and it is more toxic and has greater hydrological mobility than arsenate. The redox reactions between these two compounds can be achieved by chemical means, but also by some microorganisms that possess detoxification pathways (Mukhopadhyay et al., 2002; Lloyd, 2003).

Arsenic is released as waste by mining, glass manufacture, and by the computer chip and semiconductor industries, as well as from mineral debris, wood preservatives, insecticides, rodenticides, herbicides, and some other types of agrochemicals. Some arsenic compounds are used in medicine to treat typanosomiasis and leukemia, and recently, as a chemotherapeutic

agent (Mukhopadhyay et al., 2002; Lloyd, 2003; Rodriguez et al., 2003).

Human exposure to arsenic compounds can be oral or by inhalation. Oral exposure is due mainly to contaminated food, since soil contamination by arsenate can cause phytotoxicity, leading to biomagnification, but it can also be water borne. Inhalation may occur during the smelting of minerals, or when agricultural pesticides are used. Both ingestion and inhalation of arsenic compounds can cause cancer, the former, of the kidneys, liver and skin, and the latter, of the lungs. Arsenic exposure is also related to neuropathy and peripheral vascular disorders (Rodriguez et al., 2003). The study of mechanisms for arsenic decontamination has recently become increasingly relevant, as several regions of the world have suffered severe contamination of soil and water by arsenic compounds, and this is now an important public health issue. Well water in West Bengal (India) and Bangladesh, and drinking water in different regions in the USA, as well as in industrial and mining areas in various parts of the world, have been contaminated by arsenic compounds, and this is a serious menace for human health (Mazumder et al., 2000; Mukhopadhyay et al., 2002; Roychowdhury et al., 2003).

Arsenic resistance in bacteria is conferred by mechanisms involving proteins encoded by the *ars* operons (Carlin et al., 1995). The two most common types of these operons contain either five (*ars*RDABC) or three genes (*ars*RBC). The *ars*RDABC operons are found in the plasmids of Gram-negative bacteria, such as *E. Coli* R773, and *ars*RBC is generally in the plasmids of Gram-positive bacteria such as *Staphylococcus aureus* pI258, or in the bacterial chromosome (Owolabi and Rosen, 1990; Sofia et al., 1994; Diorio et al., 1995; Bruhn et al., 1996). The *ars*DR genes encode *trans*-acting proteins that are responsible for operon regulation (Wu and Rosen, 1991, 1993a,b; Chen and Rosen, 1997). *ars*R controls the basal levels of expression in response to arsenite, and *ars*D controls the upper and basal levels of expression. Arsenate and phosphate are chemically similar, which allows the arsenic ion to utilize phosphate transport systems to enter the cell. The resistance mechanism controlled by the *ars* operon comprises a specific efflux pump that removes As(III) from the cytoplasm. The specific efflux pump in most plasmids of Gram-negative bacteria is a two-component ATPase complex coded by the *ars*A and *ars*B genes (Rosen et al., 1988; Wu et al., 1992; Gatti et al., 2000). *ars*B is the potential-driven membrane arsenite efflux protein that is associated with the *ars*A gene product, a soluble ATPase subunit, to form a complex responsible for the removal of arsenite throughout the membrane. In the Gram-negative bacterial chromosome, as well as in the plasmids and chromosomes of Gram-positive bacteria, the arsenic operons do not possess the *ars*A gene and the operons have only the *ars*RBC genes (Dey and Rosen, 1995). In these cases, the *ars*B protein is solely responsible for arsenite removal from the cell.

Prior to efflux, arsenate is reduced to arsenite by an arsenate reductase, the gene product of *ars*C (Gladysheva et al., 1994; Oden et al., 1994). Bacterial arsenate reductases can be grouped into two families according to their structure and consequent catalytic mechanisms, but both share a need to be associated with proteins that promote thiol oxidation/reduction, such as glutaredoxin (Grx) or thioredoxin (Trx), in order to catalyze disulfide bond reduction (Mukhopadhyay et al., 2002). The best-studied group is the *E. coli* resistance plasmid R773 family. This arsenate reductase is dependent on Grx and reduced glutathione (GSH) to convert arsenate to arsenite. Cys12 is the amino acid residue that is thought to be the catalyst site, and some crystallographic evidence indicates the formation of two intermediates, (Cys12) S-As(V) and (Cys12) S-As(III) (Martin et al., 2001). A third intermediate in the catalytic cycle, (Cys12) S-As(V)-S (GSH), has also been proposed.

The first step in the catalytic cycle involves the formation of a thioarsenate binary adduct (Cys12) S-As(V). This compound reacts with GSH to form the (Cys12) S-As(V)-S (GSH) intermediate. In the next step of catalysis, arsenate is reduced to arsenite, and Grx is the electron donor, forming a novel covalent As(III) intermediate, (Cys12) S-As^{+}-OH^{-}, which is hydrolyzed, releasing As(III). The crystal structure of R773 arsenate reductase indicates that the amino acid residues Arg60, Arg94 and Arg107 are close to the catalytic Cys12 (Martin et al., 2001). The concentration of positive charge stabilizes the bound substrate residue and lowers the pKa.

In the *Staphylococcus* Trx family, the *S. aureus* plasmid pI258 arsenate reductase has the PTPase I-fold that is typical of low-molecular weight tyrosine phosphatases (Zegers et al., 2001). In this *ars*C enzyme, in addition to Cys10 (the equivalent cysteine residue of R778's Cys12), there are two other cysteine residues involved in catalysis, Cys82 and cys89. The active site Cys10 attacks arsenate nucleophylically, with protonated oxygen from Asp105. Cys10 provides electrons for the reduction of arsenate to arsenite, and forms a disulfide bond with cys82. Cys89 then attacks cys82, reforming the Cys10 thiolate in a step that utilizes Trx. *Bacillus subtilis* arsenate reductase also belongs to this family, and it has a similar catalytic mechanism (Bennett et al., 2001). With the help of Asp105, Cys10 thiolate attacks arsenate, forming an arsenylated enzyme-substrate intermediate, and releasing a water molecule. Asp105 functions as an acid, facilitating water release and stabilization of the intermediate that is formed. In the next step, cys10, cys82 and cys 89 are involved in a triple cysteine redox relay system to produce (Cys82) S-S(Cys89) disulfide ion, reducing arsenate to arsenite. In this step, Cys82, which is stabilized by Arg16, attacks the arsenylated intermediate, and arsenate is reduced to arsenite by transfer of two electrons from Cys10, while Cys82 forms a disulfide bond with cys89. Arg16 is responsible for lowering the pKa of the cysteine residues, activating them for reaction. The disulfide bond is reduced by Trx to regenerate the system (Bennett et al., 2001).

Chromobacterium violaceum possesses a chromosomal arsenic resistance operon of the *ars*RBC type (ORFs CV2438, CV2439 and CV2440) that comprises a regulatory protein *ars*R, a membrane-bound protein responsible for arsenite efflux *ars*B, and an arsenic reductase, *ars*C (Vasconcelos et al., 2003). The amino acid sequence of the *ars*R protein from *C. violaceum* was compared with four sequences of other *ars*R proteins found in the NCBI database, following alignment with the Clustal W program (Figure 1). The *ars*R sequence of *C. violaceum* did not contain the conserved cysteine residues (ELCVDCL), which are considered to be the metal binding domain present in typical *ars*R proteins, such as chromosomal *E. coli ars*R (Shi et al., 1994, 1996). The absence of this domain places this protein in the atypical group of *ars*R proteins, which is represented by the *ars*R protein from *Acidithiobacillus ferrooxidans* (Butcher and Rawlings, 2002). The *C. violaceum ars*R protein is most similar to a hypothetical protein from *Ralstonia metallidurans* (58% homology, E-value of 8E-28). The putative regulator *ars*R found in the NCBI that is most similar to *C. violaceum* is from *Ralstonia solanacearum* (54% homology, E-value of 9E-24). Although the binding motif is absent in these atypical proteins, other conserved residues appear in this group of regulator proteins, such as two cysteine residues close to the C-terminal of the protein and the conserved Gly-X-L-(A/G) motif located immediately downstream of the region corresponding to the metal-binding motif of typical *ars*R proteins. As they do not possess the metal binding motif, the way in which regulation is performed by these proteins is not yet well established. However, in *A. ferrooxidans*, the *ars*R protein is able to regulate the expression of the operon by responding to either As(III) or As(V) (Butcher and Rawlings, 2002).

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        5         15         25         35         45         55
C.violaceum         ----MEMKSA VTLLAALAQD TRL-AYRLLV QQGPEGLAVG QIGERLAVAN ATLSFHLKEL
A.ferrooxidans      MEPLQDPAQI VARLEALASP VRLEIFRLLV EQEPTGLVSG DIAEHLGQPH NGISFHLKNL
R.solanacearum      ----MEETDA IRSLAALAHS LRLRVFRMLV VAGPAGLTPG AIAEQLDVPG ATLSFHLKEL
E.coli ch.          ----MSFLLP IQLFKILADE TRLGIVLLLS ELG--ELCVC DLCTALDQSQ PKISRHLALL

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        65         75         85         95        105        115
C.violaceum         SHAGLILSRQ EGRFIYYSAN YEQMNALLGF LTENCCRG-E TCT------- -PANNVPPCD
A.ferrooxidans      QHAGLVTVQR EGRYQRYRAA MPVVRALVAY LTENCCHGTR DCA------L SGETRSPSVQ
R.solanacearum      MNAALVTQER DGRHLIYRAA FDHMNGLLGF LTENCCQG-E AC-------- -PEFSAAGCK
E.coli ch.          RESGLLLDRK QGKWVHYRLS -PHIPSWAAK IIEQAWRCEQ EKVQVIVRNL ARQNCSVDSK

                    ....

C.violaceum         GTC-
A.ferrooxidans      EGNQ
R.solanacearum      --C-
E.coli ch.          NTCS
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Figure 1. Sequence alignment of a selection of *ars*R proteins isolated from the chromosomes of different organisms, including *Chromobacterium violaceum*, *Acidithiobacillus ferrooxidans*, *Ralstonia solanacearum* and *Escherichia coli*. Sequences were obtained from the NCBI data bank. Boxed residues are conserved and are possibly directly involved in regulation. The alignment was performed using the CLUSTAL W-based optimal alignment tool.

A multisequence alignment was made of the *ars*C protein of *C. violaceum* and a comparison was made with other species (Figure 2). Arsenate reductase from *C. violaceum* possesses the conserved residue, Cys12, which is the active site in the *E. coli* R778 and *S. aureus* pI258 plasmids. The *C. violaceum ars*C protein also has two other cysteine residues, Cys84 and Cys92, which correspond to the conserved Cys82 and cys89 present in R773, and in the *B. subtilis* chromosome. The presence of these three conserved cysteine residues suggests that the *ars*C protein of *C. violaceum* belongs to the *Staphylococcus* Trx family. *C. violaceum* also possesses conserved Aps108 (Asp105 in *B. subtilis*) and Arg18 (Arg16 in *B. subtilis*); therefore, these residues may also be involved in the catalytic cycle.

Bacteria that possess an *ars* operon, such as *C. violaceum*, may have biotechnological applications in bioremediation processes, such as the recycling of CCA (chromated copper arsenate)-treated wood waste (Kartal and Clausen, 2001). CCA is a popular wood preservative. Used CCA-treated wood can be reutilized, recycled, incinerated, used as fuel, and can be disposed of in landfills. The recycling of treated wood into composites is an economically viable alternative to disposal into the environment, although concerns over CCA pollution limit the use of this option. Recycling of CCA-treated wood requires controlled removal of its heavy metals. One method is chemical extraction with oxalic acid; but this option lowers the quality of the composites since this very powerful acid reduces the pKa down to ~1. An alternative method is bacterial fermentation. Bioremediation of CCA-treated wood is a promising method of safely reusing treated fiber. This method takes advantage of the fact that bacteria are able to tolerate toxic levels of heavy metals, while changing them into water-soluble forms.

ACID DEHALOGENASE

Halogenated compounds are extremely toxic, and they are responsible for accelerating environmental degradation. There are a variety of halogenated compounds, and their toxicity is

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        5         15         25         35         45         55
C.violaceum         MNQPTANVLF LCTGNSCRSI LAEAVFNHLA PAGWHAMSAG SQPTGQVHPR SLALLTREGI
E.coli R773         MSNITIYHNP ACG------- TSRNTLEMIR NSGTEPTIIL YLENPPSRDE LVKLIADMGI
S.aureus pI258      MDKK--TIYF ICTGNSARSQ MAEGWGKEIL GEGWNVYSAG IETHG-VNPK AIEAMKEVDI
A.ferrooxidans      MKTP--EILF LCTGNSCRSI LAEVTFNALA GPGMHATSAG SHPAGYVHTR SINLLEREGF
P.aeruginosa        MR-----VLF MCTANSCRSI LSEAMFNHLA PPGFEACSAG SQPSGRVHPR SLATLEQAGI
P.syringae          MT-YTLFGIK ACD------- TMKKARTWLD EHSVSYEFHD YKTQAIDREH LTQWCDEHGW
B.subtilis          MENK--IIYF LCTGNSCRSQ MAEGWAKQYL GDEWKVYSAG IEAHG-LNPN AVKAMKEVGI

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        65         75         85         95        105        115
C.violaceum         ATDGYRSKSW DDLP-VTPDI VITVCASAAG ETCPVFLGPA LRSHWGVDDP AHAKGSNEEI
E.coli R773         SVRALLRKNV EPYE--QLGL AEDKFTDDQ- ----LID-FM LQHPILINRP IVVTPLG---
S.aureus pI258      DISNHTSDLI DNDILKQSDL VVTLCSDAD- NNCPILPPNV KKEHWGFDDP AGKEWS----
A.ferrooxidans      RTDGLHSKSW EDLK-ETPDI VITVCADAAG ETCPAYLGPA IRTHWGVEDP AKVTGTEAQI
P.aeruginosa        ATHGLYSKGS EAFEGAPPDI VITVCDAAAG EACPLYLGAA LKAHWGLADP SALDGDEALR
P.syringae          QV--VLNRAG TTFR--KLDD GQKADLDQA- ----KAIELM LAQPSMIKRP VLDLGN----
B.subtilis          DISNQTSDII DSDILNNADL VVTLCGDAA- DKCPMTPPHV KREHWGFDDP ARAQGTEEEK

                    ....|....| ....|....| ....|....| ....|....| ....|.
                       125        135        145        155        165
C.violaceum         DAAFLAAYRI LRARIEAFLA LPLDTLRE-D PLRLKTELER IGTLHR
E.coli R773         -TRLCRPSEV VLDILQDAQK GAFTKEDG-- ----EKVVDE AGKRLK
S.aureus pI258      -EF------- --QRVRDEIK LAIEKFK--- ---LR----- ------
A.ferrooxidans      EAAFDTAYHI LRHRIEALLQ LPVAELLEKD PAKLRQELER IGTLLP
P.aeruginosa        DAAFHATLAR IEQRCRAFLG LPFATLDR-D --QLKRELER IGSL--
P.syringae          --------KT LIGFKPDLYA AALQ------ ---------- ------
B.subtilis          WAFF------ --QRVRDEIG NRLKEFAETG ----K----- ------
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Figure 2. Sequence alignment of several arsenic reductases (a*rs*C) isolated from different organisms, including *Chromobacterium violaceum*, *Escherichia coli* plasmid R773, *Staphylococcus aureus* plasmid pI258, *Acidithiobacillus ferrooxidans*, *Pseudomonas aeruginosa* PA01, *Pseudomonas syringae*, and *Bacillus subtilis*. Sequences were obtained from the NCBI data bank. Boxed residues are conserved and are possibly directly involved in catalysis. The alignment was made using the CLUSTAL W-based optimal alignment tool.

related to the position and the number of halogen atoms linked to organic compounds. There are two main sources of halogenated compounds: anthropogenic activities (primarily industrial incineration and forest fires), and metabolites released by living organisms. Volcanic activity can also result in the release of halogenated compounds into the environment. The main concern is a tendency for the bioaccumulation of brominates, fluorinates and especially, chlorinated dioxin compounds, which are extremely toxic and carcinogenic in humans (Bunge et al., 2003).

The problem with chlorinated dioxins is the high production rates, and their high hydrophobicity, which causes precipitation of this toxic element in aquatic sediments, and as a consequence, can result in high levels of biomagnification (Bunge et al., 2003).

Some enzymatic processes are capable of reducing the toxicity of halogenated compounds. The most important enzyme involved in these processes is acid dehalogenase. Some organisms, such as bacteria, not only produce halogenated compounds, or utilize them as growth substrates, but they also possess acid dehalogenase, which reduces the toxicity of halogenated compounds by cleaving the halogen atom from organic compounds, through a process called dehalogenation (Janssen et al., 2001). There are several enzymatic mechanisms that can act on halogenated compounds, including hydrolytic, thiolytic, oxidative, and reductive dehalogenation. The most common process is hydrolytic dehalogenation, which involves substitution of the halogen atom by a hydrogen or a hydroxyl group from water, resulting in the formation of a new, less-toxic, compound (Van Pée and Unverucht, 2003). *C. violaceum* has an ORF (CV0864)

that encodes an acid dehalogenase. The sequence of this ORF exhibits a high degree of similarity with *Pseudomonas aeruginosa* (51% homology, E-value 3E-60) (Vasconcelos et al., 2003). Studies of *Pseudomonas*, *Moraxella* sp. and *Xanthobacter autotrophicus* have revealed hydrolytic dehalogenation of L-2-haloacids, which produces the corresponding, but less toxic, D-2-hydroxy acids by L-2-haloacid dehalogenase catalysis (Hisano et al., 1996). The crystal structure of L-2-haloacid dehalogenase of *Pseudomonas* sp. YL was determined at 2.5 Å resolution; this enzyme is formed by two domains: a core domain with an α/β type structure, which consists of six parallel β-sheets (β1, and β4-β8), flanked on both sides by five α-helices (α5-α9), and a sub-domain, which is composed of four α-helices (α1-α4), next to each other. This protein has nine conserved amino acid residues: Asp10, Thr14, Arg41, Ser118, Lys151, Tyr17, Ser175, Asn177, and Asp180. When the conserved amino acid residues Asp10, Thr14, Arg41, Lys151, Tyr17, Ser175, Asn177, and Asp180 were replaced by other amino acids, a significant decrease of the Vmax value for the dehalogenation reactions was recorded, indicating that these amino acids are involved in catalysis (Kurihara et al., 1995). Except for Arg-41, all of these residues are found in the core domain, forming the cleft between the core and sub-domains, that is, the active site of the enzyme (Hisano et al., 1996). The amino acid sequence alignment of the acid dehalogenase from *C. violaceum* and five different *Pseudomona* spp. (*P. aeruginosa*, *P. putida*, *P.* sp., *P.* sp. YL, and *P. syringae*) showed that seven of the nine amino acid residues (Asp10, Thr14, Arg41, Ser175, Lys151, Tyr17, Asp180) are conserved in all species (Figure 3). The amino acid residues Asn177 and Ser118, both present in the loop between β6 and α8, are changed to His and Ala in *P. aeruginosa* and *C. violaceum*. In *P*. sp. YL, the replacement of Ser118 by alanine caused an increase in the *K*m value for L-2-chloropropionate, suggesting that this amino acid is involved in substrate binding (Kurihara et al., 1995), which probably also occurs in *C. violaceum* and *P. aeruginosa*. The conservation of the seven amino acid residues in all *Pseudomonas* and *C. violaceum*, and the equivalent replacement of the remaining two amino acids in both *P. aeruginosa* and *C. violaceum*, suggest that the same hydrolytic dehalogenation mechanism occurs in both genera.

Since haloacid dehalogenase is often involved in detoxification of xenobiotics or metabolic products (Janssen et al., 2001), the finding of ORF (CV0864) in the *C. violaceum* genome, which encodes an acid dehalogenase, should be tested for enzymatic biodegradation activity, as it may have a highly exploitable biotechnological application.

THE *CYN* OPERON

Cyanate can occur in nature at high concentrations (~1 mM), for example as a result of the photooxidation of cyanide. Toxicity to the cell arises from its reactivity with nucleophilic groups in proteins (-SH, NH_2, -OH and COOH). In *E. coli*, cyanase is an inducible protein produced in response to extracellular cyanate. This enzyme catalyses the reaction between cyanate and bicarbonate, producing two CO_2 molecules: $NCO^- + 3H^+ + HCO_3 \rightarrow 2CO_2 + NH_4^+$. Cyanase is one of the three gene products encoded by the *cyn* operon. This operon includes *cyn*T, *cyn*S and *cyn*X. *cyn*T encodes a carbonic anhydrase, *cyn*S cyanase and *cyn*X encodes a hydrophobic protein of unknown function (Sung and Fuchs, 1998; Guilloton et al., 1992).

Carbonic anhydrase prevents cellular depletion of bicarbonate during cyanate decomposition (the CO_2 gas diffuses across the cell faster than the non-catalyzed hydration reaction

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45         55
C.violaceum      ---------- ---MSQPVQA LLFDVFGTVV DWREG----- ---VARDAAA FLRRRPAARQ
P.aeruginosa     ---------- -------MRA ILFDVFGTLV DWRSS----- ---LIEQFQA LER-ELGGTL
P.sp.YL          ---------- ----MDYIKG IAFDLYGTLF DVHSV----- ---VGRCDEA FPG-------
P.fluorescens    ---------- ----MKNIQG AVFDLYGTLY DVNSV----- ---AQVCEEV YSG-------
P.putida         ---------- ----MQPIEG IVFDLYGTLY DVHSV----- ---VQACESA YPG-------
P.sp.            ---------- ----MQEIRG VVFDLYGTLC DVHSV----- ---AQLCGQY FPE-------

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     65         75         85         95        105        115
C.violaceum      ------DAYA FADAWRALYS PAMEAVRAGR RPFTRLDQLH RENLEATLPA FGVDPASA-P
P.aeruginosa     ------PCVE LTDRWRQQYK PAMDRVRNGQ APWQHLDQLH RQSLEALAGE FGLALD----
P.sp.YL          ------RGRE ISALWRQK-Q LEYTWLRSLM NRYVNFQQAT EDALRFTCRH LGLDLD----
P.fluorescens    ------HGDS ISRLWRQK-Q LEYTWLRSLM GRYVNFEKAT EDALRFTCTH LGLSLD----
P.putida         ------QGEA ISRLWRQK-Q LEYTWLSSLM GRYASFEQRT EEALRYTCKH LGLATD----
P.sp.            ------RGTE ISLMWRQK-Q LEYSWLRSLM GQYVSFPQAT EDALVFVCNA LNLKLR----

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    125        135        145        155        165        175
C.violaceum      EDELQWLNRA WHRLDPWPDS VAGLARLKAG -HIIATLS-N GNTSLLLNMA KYAGLP--WD
P.aeruginosa     EALLQRITGF WHRLRPWPDT LAGMHALKAD -YWLAALS-N GNTALMLDVA RHAGLP--WD
P.sp.YL          ARTRSTLCDA YLRLAPFSEV PDSLRELKRR GLKLAILS-N GSPQSIDAVV SHAGLRDGFD
P.fluorescens    DETHQRLSDA YLHLTPYADT ADALRRLKAA GLPVGIIS-N GSHCSIEQVV TNSEMNWAFD
P.putida         ETTLRQLGQA YLHLAPHPDT TAALRRLKAS GLPMAIAS-N GSHHSIEQVV SHSDMGWAFD
P.sp.            EDTRIALCNE YLNIKPYREV RSALESLRSG AVPLAILS-N GSAHSIQSVV GNAGIEHFFS

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    185        195        205        215        225        235
C.violaceum      AILGAEAAQA YKPMPQAYLR TAELLDLPP- ---------E RICLVAAHNG DLAAARRCGF
P.aeruginosa     MLLCADLFGH YKPDPQVYLG ACRLLDLPP- ---------Q EVMLCAAHNY DLKAARALGL
P.sp.YL          HLLSVDPVQV YKPDNRVYEL AEQALGLDR- ---------S AILFVSSNAW DATGARYFGF
P.fluorescens    QLISVEDVQV FKPDSRVYSL AEKRMGFPK- ---------E NILFVSSNAW DASAASNFGF
P.putida         HLISVETVKV FKPDNRVYSL AEQTMAIPR- ---------D RLLFVSSNSW DATGARHFGF
P.sp.            HLISADEVSV SKPSPAAYEL AEKRLKVVR- ---------S KLLFVSSNAW DASGARHFGF

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    245        255        265        275        285        295
C.violaceum      K----TAFIM RPREFGPEQR TDLA-----A EQDWDWVARD LVELAGILGC ----------
P.aeruginosa     K----TAFIA RPLEYGPGQS QDLA-----A EQDWDLIASD LLDLHRQLAA SA--------
P.sp.YL          P----TCWIN RTG----NVF EEMG-----Q TP--DWEVTS LRAVVELFET AAGKAEKG--
P.fluorescens    P----VCWIN RQN----GAF DELD-----A KP--THVVRN LAEMSNWLVN SLD-------
P.putida         P----VCWVN RQG----AVF DELG-----A TP--TREVRD LGEMSDWLLD ----------
P.sp.            Q----VCWVN RSR----NTF EQLG-----E RP--DHVISG LDELPNLLNF ASADR-----
```

Figure 3. Sequence alignment of acid dehalogenases isolated from different organisms, including *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas* sp YL, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas* sp. Sequences were obtained from the NCBI data bank. Boxed residues are conserved and are possibly directly involved in catalysis. The alignment was performed using the CLUSTAL W-based optimal alignment tool.

back to bicarbonate). In the *cyn*T mutant strain of *E. coli*, the addition of cyanate results in depletion of cellular bicarbonate and in growth inhibition. Bicarbonate/CO_2 appears to compete with cyanate for an important, but unknown site, at which cyanate binding inhibits growth. This inhibition of growth suggests that bicarbonate/CO_2 plays an important role in *E. coli* growth, other than simply acting as a substrate for carboxylation, given that the addition of metabolites dependent on the carboxylation reaction did not reverse the inhibition (Kozliak et al., 1995).

The crystal structure of cyanase was examined at 1.65 Å resolution (Walsh et al., 2000). This protein is a homodecamer, and each monomer has two domains. The N-terminal domain is similar to the DNA-binding α-helix bundle motif. The C-terminal is an "open-fold"

with no structural homology to other known proteins. The subunits are arranged as dimers, and the decamer is formed by a pentamer of these dimers. The active site is located between dimers and residues of four adjacent subunits that participate in the active site. There are five active sites located at the interface of adjacent dimers (Walsh et al., 2000).

The proposed catalytic mechanism, based on crystallographic data of cyanase complexed with oxaloacetate and chloride, suggests that bicarbonate and cyanate interact with Ser122, an important residue in substrate binding. Cyanate and bicarbonate are negatively charged, and they are probably stabilized by Arg96, forming a salt bridge with Glu99, which interacts with a water-bound molecule. This water may protonate the bound cyanate nitrogen, making the cyanate carbon more electrophylic. The bicarbonate carboxylate oxygen attacks this carbon nucleophilically, forming an intermediate dianion, which is decarboxylated into CO_2 and carbamate (Johnson and Anderson, 1987; Walsh et al., 2000).

C. violaceum possesses a *cyn* operon encoding for *cyn*T, *cyn*S and *cyn*X. The *cyn*T amino acid sequence is most similar to carbonate dehydratase from *P. aeruginosa* PA01 (69% homology, with an E-value 3E-78). The homology of *cyn*S is highest with cyanate lyase, also from *P. aeruginosa* PA01 (75%, E-value 2E-61). The greatest homology of *cyn*T is with a hypothetical protein from *R. metallidurans* (56%, E-value of 3E-82), followed by *E. coli* K12 (54% E-value of 1E-81). The high degree of homology observed between *cyn*S proteins can be explained by their structural properties, such as the formation of decamers. The alignment of amino acid sequences from four cyanate lyases, including the cynS from *C. violaceum*, shows that the residues forming the active site are conserved for the four proteins (Figure 4).

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5          15         25         35         45         55
C.violaceum      -MQSQCQPAP RQALTDAVIA AKARKRLSFE DINQGTGLSL AYTTAALLGQ HALPEAAARL
P.aeruginosa     MQHSQVSPNA RQQLAETVVL NKARLGLSWQ DLADGTGLAL TFVTAALLGQ HALPEAAARK
E.coli K12       MIQSQINRNI RLDLADAILL SKAKKDLSFA EIADGTGLAE AFVTAALLGQ QALPADAARL
A.thaliana       -----MEAAK KQSVTNQLLA VKSASGKTFS QLAAETGLTN VYVAQLLRRQ AQLKPDTVPK

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     65         75         85         95        105        115
C.violaceum      VAERLELDED AIALLQAIPL RGSIPGGVPT DPTIYRFYEM VQIYGTTLKA LVHEQFGDGI
P.aeruginosa     VAAQLGLDDD AVLLLQSIPL RGSIPGGIPS DPTIYRFYEM LQVYGSTLKA LVHEQFGDGI
E.coli K12       VGAKLDLDED SILLLQMIPL RGCIDDRIPT DPTMYRFYEM LQVYGTTLKA LVHEKFGDGI
A.thaliana       LKEALPALTD ELIGDMMSPP WRSYDPNLIQ EPTIYRLNEA VMHFGESIKE IINEDFGDGI

                 ....|....| ....|....| ....|....| ....|....| ....|....| ...
                    125        135        145        155        165
C.violaceum      ISAINFKLDI RKVEDPDGGH RAVITLDGKY LPTKPF---- ---------- ---
P.aeruginosa     ISAINFKLDI KKVEDPEGGS RAVITLDGKY LPTKPF---- ---------- ---
E.coli K12       ISAINFKLDV KKVADPEGGE RAVITLDGKY LPTKPF---- ---------- ---
A.thaliana       MSAIDFYCSV DKIKGVDGNN RVVVTLDGKY LSHSEQRTEN MVSRLNLKGG TSE
```

Figure 4. Sequence alignment of cyanate lyase (cyanase-cynS) isolated from different organisms, including *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Arabidopsis thaliana*. Sequences were obtained from the NCBI data bank. Boxed residues are conserved and are proposed active-site residues. The alignment was performed using the CLUSTAL W-based optimal alignment tool.

The ammonia produced in the reaction catalyzed by cyanase can be utilized by these bacteria as a source of nitrogen (Guilloton et al., 1992; Kozliak et al., 1995). The metabolization of toxic compounds, like cyanate, to compounds that can be used in structural cell functions, has potential biotechnological applications in environmental detoxification.

CYANIDE AND GOLD RECOVERY

Hydrogen cyanide is a secondary metabolite formed by a restricted group of bacteria. HCN is a potent inhibitor of cytochrome oxidase and several other metalloenzymes, some of them involved in respiratory processes. HCN biosynthesis is catalyzed by HCN synthase, from glycine, with stoichiometric production of CO_2. Apparently, this enzyme is a membrane-bound protein, sensitive to molecular oxygen, which made its purification difficult (Castric, 1994). In extracts, HCN synthase from *Pseudomonas* sp. oxidizes glycine in the presence of artificial electron acceptors, such as phenazine methosulfate (Wissing, 1974). Optimal expression of HCN synthase is obtained at low oxygen levels, in the transition from the exponential to the stationary phase (Castric et al., 1979). The structural genes encoding HCN synthase are *hcn*ABC. These genes were already found to be expressed by the T7 promotor in *E. coli*, resulting in HCN production by this bacterium (Laville et al., 1998). Analysis of nucleotide sequences showed that each of the HCN synthase subunits has similarities with known enzymes, such as formate dehydrogenase with *hcn*A, and amino acid dehydrogenases with *hcn*B and *hcn*C (Laville et al., 1998). The sequence comparisons suggest that HCN synthase is a complex with amino acid oxidase functions. Partially purified HCN synthase indicates the presence of an FAD co-factor, which is in agreement with other biochemically characterized amino acid oxidases. The catalytic mechanism involving HCN synthase is not well known, and so far there is no structural data available for the enzyme; however, a catalytic mechanism was proposed, based on nucleotide sequence analysis of the *hcn*ABC operon, similar to the dehydrogenase model. Glycine is initially oxidized to iminoacetic acid, and then the C-C is split in a second dehydrogenase reaction, producing HCN and CO_2. The difference between this mechanism and the classical mechanism of D- or L-amino acid oxidases is the splitting of the C-C bond of the iminoacetic acid intermediate, instead of being hydrolytically converted to glyoxylic acid, which is the traditional product of the amino acid oxidases. Cleavage of iminoacetic acid by HCN synthase could produce HCN and formic acid, which in turn could be oxidized to CO_2 by the *hcn*A subunit. Four electrons are transferred from glycine, probably to a cyanide resistant oxidase. *hcn*B and *hcn*C possess two transmembrane segments, in agreement with the proposal of HCN synthase being a membrane-bound protein (Laville et al., 1998).

Products of virulent factors and secondary metabolites are under quorum-sensing control in *P. aeruginosa*, a mechanism based on the coordination of gene expression dependent on cell density. This coordination is performed by N-acylhomoserine lactone (AHL)-mediated quorum sensing, which also exists in *C. violaceum* (McClean, 1997). Two signal molecules, N-(3-oxo-dodecanoyl)-homoserine lactone produced by *Las*I enzyme and N-butanoyl-homoserine lactone produced by *Rh1*I enzyme, interact with *Las*R and *Rh1*R, two transcriptional regulators that activate the expression of genes involved in hydrogen cyanide production. The production of N-butanoyl-homoserine lactone in the cell is under the control of the GacS/GacA system, and although this mechanism is still partially unknown there is evidence that GacA has a positive effect on LasR and RhlR transcription. The GacS/GacA regulatory system controls the secondary metabolism levels, upregulating the *hcn* promotor by activation of RhlR, in a quorum sensing modulating mechanism, and at a second level it has a positive effect on *hcn* translation, probably involving the RNA-binding protein RsmA (Pessi and Haas, 2000, 2001; Pessi et al., 2001).

Cyanide is used in the production of nitrile, nylon, synthetic rubber, and acrylic plastics, as well as for steel hardening, metal processing, and in photographic applications (Akcil, 2003).

Cyanide has been used in gold mining since the end of the 19th century. Gold is a precious metal that occurs in nature in its native form, usually associated with quartz. Such ores are treated in a conventional process with cyanide (cyanide heap leach process) to solubilize the gold-cyanide complex. In the cyanide heap leach process, the ore is crushed and placed on pads lined with compacted clay or polyethylene sheets (Korte et al., 2000). A diluted cyanide solution (0.05%) percolates through the heap, dissolving the gold. The leaching process can take several days or months, depending on the procedure and the ore. The cyanide-metal complex formed is stored in ponds. The liquid undergoes several processes to remove the gold, such as activated charcoal treatment, heat, chemical processes, or electrolysis (Korte et al., 2000). These treatments are difficult, since metals are complexed or changed in the oxidation state by biological systems and the effects are difficult to predict. However, their ability to drastically alter the environmental pH has contributed to the employment of some bacteria for bioleaching, which is currently used in the mining of gold. Bioleaching is a new technique used in the mining industry to extract minerals such as gold from their ores; it minimizes some of the associated environmental problems, such as cyanide leaks from the leach processing and open pile and solution ponds, which can overflow and spill cyanide into the environment. The heap leach processes have additional environmental problems, including the release of associated heavy metals, present in the ores. Bacterial oxidation has the advantage of reducing the capital costs and simultaneously reduces the environmental contamination associated with gold recovery.

Chromobacterium violaceum has the *hcn*ABC operon responsible for HCN production. The most similar amino acid sequence in NCBI to the *C. violaceum hcn*A (ORF CV1684), *hcn*B (ORF CV1683), and *hcn*C (ORF CV1682) is hydrogen cyanide synthase from *P. fluorescens* (72%, E-value 1E-20 to *hcn*A; 66%, E-value 0.0 to *hcn*B, 79%, E-value 0.0 to *hcn*C).

The utilization of *C. violaceum* in gold recovery has already been tested (Campbell et al., 2001). Campbell et al. showed that *C. violaceum* grown in nutrient broth formed a biofilm and could complex and solubilize up to 100% of the gold on glass test slides, within less than a week. This bacterium also has the ability to mobilize gold from a bioxidized sulfidic ore concentrate. The utilization of inexpensive agricultural feedstocks instead of commercial bacterial media is also possible, and it decreases the cost of the process.

CONCLUDING REMARKS

In many environments, pollutants, such as organic compounds and heavy metals, are very problematic. Both bioleaching (the extraction of specific metals from their ores with bacteria) and bioremediation (the controlled degradation or transformation of hazardous or toxic substances by microbial organisms) are potential areas in which biotechnology could act. The three sets of genes related to arsenic resistance, cyanate degradation, and acid dehalogenation, found in the *C. violaceum* genome could be explored biotechnologically for both environmental pollution control and bioremediation. The pathway of interest and the associated genes were studied in detail, and the molecular interactions involved in their function have been described. We now need to go further and modify the system (through gene deletion or overexpression), monitor the changes in expression patterns and combine the observations on expression patterns with existing data. The tools that have been developed for genetic manipulation make such genes attractive for bioremediation.

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