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Whole-genome analysis of *Azoarcus* sp. strain CIB provides genetic insights to its different lifestyles and predicts novel metabolic features

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Abbreviations: ANI, average nucleotide identity; BIMEs, bacterial interspersed mosaic elements; IAA, indoleacetic acid; ICE, integrative and conjugative element; REPs, repeated extragenic palindrome sequences; ROS, reactive oxygen species; TAS, toxin-antitoxin system; TMAO, trimethylamine *N*-oxide.

1 **ABSTRACT**

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4 The genomic features of *Azoarcus* sp. CIB reflect its most distinguishing phenotypes as
5 a diazotroph, facultative anaerobe, capable of degrading either aerobically and/or
6 anaerobically a wide range of aromatic compounds, including some toxic hydrocarbons
7 such as toluene and *m*-xylene, as well as its endophytic lifestyle. The analyses of its
8 genome have expanded the catabolic potential of strain CIB towards common natural
9 compounds, such as certain diterpenes, that were not anticipated as carbon sources. The
10 high number of predicted solvent efflux pumps and heavy metal resistance gene clusters
11 has provided the first evidence for two environmentally-relevant features of this
12 bacterium that remained unknown. Genome mining has revealed several gene clusters
13 likely involved in the endophytic lifestyle of strain CIB, opening the door to the
14 molecular characterization of some plant growth promoting traits. Horizontal gene
15 transfer and mobile genetic elements appear to have played a major role as a mechanism
16 of adaptation of this bacterium to different lifestyles. This work paves the way for a
17 systems biology-based understanding of the abilities of *Azoarcus* sp. CIB to integrate
18 aerobic and anaerobic metabolism of aromatic compounds, tolerate stress conditions,
19 and interact with plants as an endophyte of great potential for phytostimulation and
20 phytoremediation strategies. Comparative genomics provides an *Azoarcus* pan genome
21 that confirms the global metabolic flexibility of this genus, and suggests that its
22 phylogeny should be revisited.
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38 *Keywords:* *Azoarcus*, aromatic compounds, endophyte, metals resistance, mobile genetic
39 elements, comparative genomics
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Introduction

Azoarcus is a genus of betaproteobacteria that belongs to the family *Rhodocyclaceae*, a physiologically versatile group encompassing bacteria with diverse functions [60]. The environmental relevance of *Azoarcus* strains is supported by their frequent detection in diverse soils, sludges, and wastewaters [39]. The *Azoarcus* genus, that includes ten recognized species, namely *A. indigenus* (type species of the genus), *A. communis*, *A. toluelyticus*, *A. toluvorans*, *A. toluclasticus*, *A. evansii*, *A. anaerobius*, *A. buckelii*, *A. olearius*, and *A. taiwanensis* [2,31,42,49], was shown to comprise bacteria that fit into one of two major phylogenetic and eco-physiological groups [22,28,38,49]. One group includes free-living bacteria that usually inhabit waters and soils and participate in the biogeochemical cycling of a large number of organic and inorganic metabolites [31,40,43,49]. Many strains of this group have been described and/or isolated by their ability to degrade aromatic compounds in anoxic conditions, being strain EbN1 (currently "*Aromatoleum aromaticum*" EbN1) [38,39] and *A. evansii* KB740 [2,14] the two most studied. The other group includes *Azoarcus* strains such as *A. communis* strain SWub3, *A. indigenus* strain VB32 or the well-characterized *Azoarcus* sp. strain BH72, that invade roots of Kallar grass and rice, living as endophytic bacteria [42]. Interestingly, the free-living *Azoarcus* strains that are anaerobic biodegraders of aromatic compounds, and whose prototype is the EbN1 strain, have exclusively received particular attention for their degradation and biotransformation abilities [22,38,39].

Azoarcus sp. CIB (CECT#5669) was isolated from a DSMZ 12184 culture (not available anymore), which was supposed to be *Azoarcus* sp. strain M3, isolated from a diesel fuel-contaminated aquifer at Menziken (Switzerland) [20,32]. *Azoarcus* sp. CIB is a free-living bacterium with the ability to degrade a high number of aromatic compounds under aerobic and/or anaerobic conditions, including some toxic hydrocarbons such as toluene and *m*-xylene [9,25,32,57]. Recently, we have demonstrated that *Azoarcus* sp. CIB has also the ability to grow in association with plants, colonizing the intercellular spaces of the exodermis of rice roots. In addition, the strain CIB displays plant growth promoting traits such the ability to uptake insoluble phosphorous, production of indoleacetic acid (IAA) or nitrogen fixation [16]. Thus, *Azoarcus* sp. CIB may represent the prototype of a subgroup of *Azoarcus* strains that share the anaerobic biodegradation of aromatic hydrocarbons with a facultative endophytic lifestyle [16]. Since *Azoarcus* sp. CIB presents a robust growth and it is susceptible of genetic manipulation, it became a model system to study the complex regulatory networks that control the expression of the aerobic and anaerobic aromatic degradation clusters [5,9,13,57,58], and some recombinants strains have been engineered for biotechnological prospects [63].

In this work, we sequenced the whole genome of *Azoarcus* sp. CIB and accomplished a comparative analysis with the genomes of other strains, such as *Azoarcus* sp. BH72 and strain EbN1, that are the prototypes of obligate endophytes and free-living strains, respectively. This work provides new insights into the genetic determinants that may account for some of the reported metabolic abilities of the CIB strain, and offers information on genetic characteristics that may be relevant for the adoption of a particular lifestyle or that can be of biotechnological interest.

Materials and Methods

Genome sequence, contigs assembling, and gaps filling

Azoarcus sp. CIB was anaerobically grown at 30 °C in MC medium [32] containing 3 mM benzoate as sole carbon and energy source and 10 mM nitrate as electron acceptor. Cultures were collected when they reached the early stationary phase and genomic DNA was extracted using previously published protocols [32].

The genome sequencing of *Azoarcus* sp. CIB was carried out using the 454 Life Sciences high-density pyrosequencing methodology in a GSFLX sequencer from Roche at LifeSequencing (Valencia, Spain). FASTQ reads (about 250-nt long) were assembled in contigs by using the Newbler software from Roche. Contigs were ordered in scaffolds by performing a long-tag paired-end sequencing according to Roche protocols at LifeSequencing (Valencia, Spain). Gap filling on the scaffolds was performed by manual assembly of FASTQ reads with BioEdit (Ibis Biosciences) and by conventional sequencing methods (ABI Prism 377; Applied Biosystems) of PCR products (purified with Gene Clean Turbo, Q-BIOgene) spanning the regions between flanking contigs.

Gene prediction and genome annotation

The genome of *Azoarcus* sp. CIB was annotated by means of a bacterial genome annotation pipeline [56], which used tRNAscan-SE to predict tRNA genes, RNAmmer to predict rRNA genes and Glimmer to predict coding sequences. Functional annotations for proteins were generated by comparison against several protein sequence and protein family databases (SwissProt, NCBI protein, COG, Pfam, Smart, Prk) with BLAST and RPS-BLAST [1]. Annotations were summarized in different output formats. One of them was used as input for Pathway Tools, for automatic metabolic reconstruction [27].

Transposase and integrase encoding genes were manually annotated with the assistance of the ISFinder database (<http://www-is.biotoul.fr/>).

Comparative genomics

The complete genome of *Azoarcus* sp. CIB was compared with that of all currently sequenced strains: "*Aromatoleum aromaticum*" strain EbN1 (NC_006513.1; NC_006823.1; NC_006824.1); *Azoarcus* sp. BH72 (NC_008702.1); *Azoarcus* sp. KH32C (NC_020516.1; NC_020548.1); and *Azoarcus toluclasticus* strain MF63 (NZ_ARJX00000000.1). The closely related *Thauera* sp. strain MZ1T (CP001281.2, CP001282.1) was used as outgroup. Comparative genomic analyses, including Venn diagrams, synteny analyses and phylogenetic trees were performed with EDGAR [6]. Average nucleotide identity among the genomes based on MUMmer (ANIm) was calculated with Jspecies [44].

Nucleotide sequence accession number

The *Azoarcus* sp. strain CIB whole genome sequence and annotation has been deposited in GenBank and is available under accession number CP011072.

Substrate diversity studies

Azoarcus sp. CIB was tested for its ability to utilize various carbon sources in MC medium in aerobic conditions or anaerobiosis (10 mM nitrate as electron acceptor) when cultured for 48 h at 30°C. For each substrate, two replicates and a control without inoculation were included. Aromatic compounds that had not been tested previously and that allowed growth were: cinnamate and *p*-coumarate (anaerobiosis); gentisate and cumene (aerobiosis). Other aromatics that did not allow growth were: 2-hydroxybenzoate, 3-hydroxyphenylacetate, 2-hydroxyphenylacetate, 2-hydroxyphenylpropionate, protocatechuate, catechol, homogentisate, resorcinol, ferulate, vanillin, vanillate, nicotinate, isonicotinate, *o*-phthalate, mandelate, phenylglyoxylate, tyramine, 2-aminobenzoate, 4-aminobenzoate, 3-fluorobenzoate, 2-chlorobenzoate, 3-chlorobenzoate, benzene, propylbenzene, styrene, biphenyl. Non aromatic carbon sources tested that provided growth were: acetate, citrate (aerobiosis) propionate, lactate (anaerobiosis), pyruvate, butyrate, 3-hydroxybutyrate, isobutyrate, valerate, isovalerate, succinate, fumarate, malate, glutarate, adipate, pimelate, Ala, Pro, Glu, Asp, Arg, Asn, Gln, Ile, Leu, Gly, Val, His, Ser, and abietic acid (aerobiosis). Non aromatic carbon sources tested that did not allow growth were: fructose, maltose, glycerol, galactose, arabinose, saccharose, manitol, ribose, xylose, Thr, Trp, Lys, maleate, quinate, limonene, geraniol, citronellol, cyclohexanol, cyclohexanone, cholesterol, isopropanol, butanol, propanol, octanoate, decanoate.

Results and discussion

Genome organization

The complete genome of *Azoarcus* sp. CIB was constructed as a single circular chromosome consisting of 5,257,030-bp by assembling sequence data from pyrosequencing and Sanger sequencing of PCR products. A total of 872,867 sequencing reads were obtained from three DNA pyrosequencing runs and a paired-end run, providing a 42-fold coverage of the genome. The sequence was assembled into 645 contigs with an average length of 9748 bp, which were, in turn, organized in 38 scaffolds. The predicted size of the *Azoarcus* sp. CIB chromosome was 5,145,765 bp, based on those sequences. Manual curation of the assembly allowed to fill-in 349 out of 655 gaps. The remaining 306 gaps were filled-in by conventional sequencing of PCR products obtained by using primers that were designed to the ends of each of the remaining contigs. Gap filling added 111,265 bp to the initial genome assembly.

Table 1 shows basic characteristics of the genome of *Azoarcus* sp. strain CIB and their comparison with those of the other four sequenced *Azoarcus* strains, i.e., EbN1 [38], BH72 [28], KH32C [37], and MF63 (BioProject PRJNA199393). The chromosome contains 4 complete 16S-tRNA^{Ile}-tRNA^{Ala}-23S-5S operons (Fig. 1). A total of 2347 genes (49.5 % of the total protein-coding genes) of *Azoarcus* sp. CIB were assigned to a functional category of clusters of orthologous groups of proteins.

The genomic structures of *Azoarcus* sp. CIB and *Azoarcus* sp. KH32C strain were syntenic, except in the region from position 1.0 to 3.0 Mb (Fig. S1). Many of the breakpoints in synteny corresponded to the presence or absence of integrated elements. However, synteny was not extended to the EbN1 and BH72 strains (data not shown), suggesting a more distant phylogenetic relation for these two strains.

The mobilome of *Azoarcus* sp. CIB

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2 A notable genomic feature of strain CIB is the presence of an extensive
3 mobilome. Thus, the genome of *Azoarcus* sp. CIB contains a high number of full-length
4 and partial genes encoding putative transposases (85) and phage-related
5 integrases/recombinases (16) that belong to a wide variety of families and many of
6 which are paralogs (Table S1). All these genes are distributed across the chromosome
7 but appear to be particularly densely clustered at some genome hot spots (Fig. 1).
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10 Out of the 14 toxin-antitoxin systems (TASs) present in the genome of strain
11 CIB, seven are located in close proximity to genes encoding transposases (Fig. 1),
12 which suggests a major role in the maintenance of mobile genetic elements. Other TASs
13 may play important roles in cell physiology of strain CIB under stress conditions, as it
14 has been shown in other bacteria [59].
15

16 Several site-specific integrated elements have been identified in the genome of
17 strain CIB (Table 2). Some of them contain site-specific recombinases or transposases,
18 and/or they are targeted to tRNAs. Moreover, most of these integrated elements show a
19 GC content that is significantly lower or higher than the average GC content (65.8%) of
20 the *Azoarcus* sp. CIB genome. All these features suggest that these integrated elements
21 are genomic islands that were acquired by horizontal gene transfer. These genomic
22 islands are likely involved in the adaptation of *Azoarcus* sp. CIB to the use of different
23 carbon sources or the endophytic lifestyle (see below). The presence of orthologs of
24 some of the genes contained in the genomic islands of strain CIB in the genome of
25 *Azoarcus* strains BH72, EbN1, KH32C and MF63, is summarized in Table 2.
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27 Repeated extragenic palindrome (REPs) sequences, often clustered as two
28 tandem inverted copies to form bacterial interspersed mosaic elements (BIMEs) [53],
29 are present in high copy number in the genome of strain CIB (Table S2). REPa/BIMEa
30 elements are frequently associated to translation and/or transcription termination signals
31 (Table S2). We have identified two similar REPa sequences in the gene clusters for the
32 aerobic degradation of benzoate and 2-aminobenzoate in *A. evansii* [19,46], and there
33 are five REPa sequences in the chromosome of strain EbN1 [38]. Interestingly, a couple
34 of REPa elements flank transposase TnpA_{REP} (AzCIB_4604) which is associated to
35 insertion sequences of the IS200/IS605 family (Fig. 1). TnpA_{REP}, which belongs to the
36 Y1-superfamily of transposases, occurs in a variety of bacteria and appears to be
37 responsible for REP proliferation throughout their host genome [53]. The TnpA_{REP} from
38 *Azoarcus* sp. CIB constitutes the first one to be identified within the *Rhodocyclaceae*
39 family.
40

41 BIMEb elements are commonly overlapping the stop codons of adjacent genes
42 (Table S2). We have identified four REPb sequences in the gene clusters for the aerobic
43 degradation of benzoate [19] and anaerobic degradation of phenylacetate (Acc. No.
44 AJ428571) in *A. evansii*, and there are 15 REPb sequences in the genome of strain
45 MF63. Most BIMEc elements are located in intergenic regions (Table S2). The BIMEc
46 element is also present in high copy number in *Azoarcus* sp. BH72, and a truncated
47 REPc sequence (34-nt long) can be identified in the genome of strains of *Vibrio*,
48 *Variovorax*, *Rhanella*, *Rhodopseudomonas*, etc., suggesting that this element is a new
49 REP/BIME sequence widely spread in proteobacteria.
50

51 52 53 54 55 56 Phylogeny of *Azoarcus* strains

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58 Comparison of the whole genome of *Azoarcus* sp. CIB with that of other strains
59 whose genome is known, i.e., *Azoarcus toluclasticus* MF63, *Azoarcus* sp. KH32C,
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1 *Azoarcus*/"*Aromatoleum*" strain EbN1, and *Azoarcus* sp. BH72, gives average
2 nucleotide identity (ANIm) values [44] of 90.35% (percentage of genome aligned
3 73.73%), 85.96% (aligned 34.76%), 85.22 (aligned 24.72%), and 84.25% (aligned
4 15.48%), respectively. As a reference, the ANIm value between *Azoarcus* sp. CIB and a
5 strain of the closely related *Thauera* genus, *T. aminoaromatica* MZ1T strain [23], was
6 84.34% (aligned 18.47%). Therefore, these data strongly suggest that strain CIB is
7 indeed a bacterial species different to those sequenced so far. Taken into account
8 previous phylogenetic analyses of the 16S rDNA sequence of strain CIB and that of the
9 other *Azoarcus* species known so far [16,32], *Azoarcus* sp. CIB would be closely related
10 to *A. evansii*/*A. toluvorans* species, which constitute the *A. toluvorans* species group.

11 Comparative genomic analyses were performed to determine putative orthology
12 relations between the sequenced *Azoarcus* strains. A Venn diagram representing the core
13 genome and the pan genome of the *Azoarcus* genus is shown in figure 2. 1701 genes are
14 shared among the five *Azoarcus* strains. The strain CIB shares 3643 genes with strain
15 MF63, 3060 genes with strain KH32C, 2395 genes with strain EbN1, and 2360 genes
16 with strain BH72 (Fig. 2). A total of 475 genes were identified as exclusive, singletons,
17 to strain CIB.

18 A core genome tree among the five compared strains placed *Azoarcus* sp. CIB
19 closest to *A. toluclasticus* MF63, with strains BH72 and EbN1 as the most distantly
20 related to strain CIB (Fig. 3A). This result is supported by the analysis of the average
21 amino acid identities of the *Azoarcus* core genome (Fig. 3B). Taken together, these data
22 confirm the ANI analyses shown above, and suggest that the phylogeny of the *Azoarcus*
23 genus should be revisited in the near future to presumably re-classify the strains into at
24 least three different genera, i.e., a first genus comprising the strains closely related to
25 species *A. buckelii*, *A. anaerobius*, *A. taiwanensis*, and "*Aromatoluem aromaticum*", a
26 second genus comprising the strains closely related to species *A. toluclasticus*, *A.*
27 *toluclasticus*, and *A. evansii*/*A. toluvorans*, and a third genus comprising the strains
28 closely related to species *A. communis*, *A. indigenus*, and *A. olearius*. Further genome
29 analyses have been focused on genetic determinants involved in the major
30 ecophysiological capacities of strain CIB.

31 *Genes for nitrogen metabolism*

32 *Azoarcus* sp. CIB is able to fix nitrogen as a diazotroph [16], which was
33 confirmed through genome analyses by the presence of a nitrogen fixation regulon.
34 Thus, a *nif* gene cluster encoding the synthesis and maturation of the nitrogenase
35 required to fix nitrogen is closely linked to another cluster that likely encodes a RnfI
36 membrane complex driving a reverse electron flow from NADH to reduce ferredoxin
37 FdxN, which serves as the electron donor to nitrogenase [45]. Both the *nif* and *rnfI* gene
38 clusters are located within genomic island III of strain CIB (Table 2, Fig. 1). The ability
39 of *Azoarcus* sp. CIB to fix nitrogen should give this organism an advantage for survival
40 in environments poor in nitrogen, and should favor plant associations (see below). By
41 growing the strain CIB aerobically on a minimal medium containing nitrate as sole
42 nitrogen source, we have confirmed here the existence of a functional assimilatory
43 nitrate reductase in *Azoarcus* sp. CIB (data not shown). Genome analyses revealed the
44 existence of potential assimilatory nitrate (AzCIB_0744) and nitrite (AzCIB_0740-
45 0741) reductases, respectively, encoded in a *nas* gene cluster [33].

46 On the other hand, *Azoarcus* sp. CIB is able to use nitrate as terminal electron
47 acceptor in the absence of oxygen, when coupled to the oxidation of organic compounds
48 [32]. Based on genome analyses we predict a complete denitrification that generates

1 nitrogen and that involves a nitrate reductase (*nar* genes; AzCIB_2181-2184), a nitrite
2 reductase (*nir* genes, AzCIB_3596-3608), a nitric oxide reductase (*nor* genes,
3 AzCIB_1316-1320), and a nitrous oxide reductase (*nos* genes, AzCIB_2188-2193) [65].
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5 *Genes for aromatic compounds degradation*

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7 The capability of *Azoarcus* sp. strain CIB to degrade a wide variety of aromatic
8 compounds, both aerobically and anaerobically, is one of most relevant metabolic
9 features of this strain and it has been subject of several studies [9,25,32]. This metabolic
10 potential was confirmed here through genome sequence analyses. About 6% of the total
11 protein-encoding genes are related to the catabolism of aromatic compounds or
12 chemicals that generate aromatic compounds during their degradation.
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14 Three different central pathways for anaerobic degradation of aromatic
15 compounds can be identified in strain CIB. Thus, in addition to the previously reported
16 gene clusters for anaerobic degradation of benzoate (*bzd* genes) and 3-methylbenzoate
17 (*mbd* genes) through the *bzd* and *mbd* central pathways, respectively (Fig. 4) [25,32],
18 the strain CIB contains an additional gene cluster (*hbd* genes) orthologous to that
19 responsible for the anaerobic degradation of 3-hydroxybenzoate in *T. aromatica* [30],
20 and strain EbN1 [38] and, hence, likely to be devoted to 3-hydroxybenzoate degradation
21 in *Azoarcus* sp. CIB (Fig. 4).
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23 A number of peripheral pathways that converge into the anaerobic benzoyl-CoA
24 central pathway can be identified in the genome of strain CIB. The genes encoding the
25 putative peripheral pathways for the anaerobic degradation of 4-hydroxybenzoate (*hcr*),
26 phenol (*pps/ppc*), *p*-cresol (*pch/pdh1*), phenylacetate/4-hydroxyphenylacetate (*pad*), 2-
27 phenylethylamine (*pea*), phenylalanine (*pat/pdc*), 2-phenylethanol (*ped*),
28 phenylacetaldehyde (*pdh/aor*), phenylpropionate/cinnamate/*p*-coumarate (*cou*) are
29 found distributed along the *Azoarcus* sp. CIB chromosome (Fig. 4) [4, 9, 38, 39]. The
30 *cou* genes are also responsible of funneling 3-hydroxyphenylpropionate to the *hbd*
31 central pathway (Fig. 4). Interestingly, a gene cluster orthologous to the *bss/bbs* cluster
32 encoding the anaerobic peripheral pathway that funnels the aromatic hydrocarbons
33 toluene and *m*-xylene to the *bzd* and *mbd* central pathways, respectively [9,25,29], has
34 been found within a putative integrative and conjugative element, ICE_{XTD}, in the
35 genome of *Azoarcus* sp. CIB (Figs. 1 and 4, Table 2).
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37 *Azoarcus* sp. CIB is also able to degrade aromatic compounds under aerobic
38 conditions [32]. The aerobic degradation of benzoate via the benzoyl-CoA hybrid
39 pathway is encoded by the *box* gene cluster [57]. Genome mining revealed a number of
40 additional gene clusters likely encoding different central pathways for the aerobic
41 degradation of aromatic compounds. The *paa* gene cluster encoding a second aerobic
42 hybrid pathway, i.e., the phenylacetyl-CoA pathway for the aerobic degradation of
43 phenylacetate [52], was identified (Fig. 4). On the other hand, gene clusters orthologous
44 to those encoding a catecholic *meta*-cleavage pathway for aerobic degradation of
45 toluene/cumene (*tod*) [10], and the central gentisate pathway for 3-hydroxybenzoate
46 degradation (*nag*) [64], were also present in the chromosome of strain CIB (Fig. 4). It
47 should be noted that the *tod* genes are also present within the ICE_{XTD} element,
48 suggesting that the ability of strain CIB to degrade either aerobically or anaerobically
49 toxic aromatic hydrocarbons has been acquired by horizontal gene transfer. Genes that
50 might encode peripheral pathways that channel other aromatic compounds, e.g., 2-
51 phenylethylamine (*pea*) and 3-hydroxyphenylpropionate (*cou*), to the *paa* and *nag*
52 aerobic central pathways, respectively, are shared with the corresponding anaerobic
53 degradation pathways (Fig. 4).
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1 In bacteria, some cyclic non-aromatic compounds, e.g., cyclohexane
2 carboxylate, diterpenes, steroids, are channeled to aromatic central pathways through
3 their degradation [18,34,48]. *Azoarcus* sp. CIB is able to degrade cyclohexane
4 carboxylate [5], and genes homologous to the *bad* genes responsible of bacterial
5 cyclohexane degradation via an alternative anaerobic central pathway [9,15] have been
6 identified in the strain CIB (Fig. 4). On the other hand, we have confirmed here (see the
7 "Substrate diversity studies" section in Materials and Methods) that the CIB strain uses
8 abietic acid, a model diterpene, as sole carbon source under aerobic conditions. It is
9 known that the *dit* genes are responsible for diterpenes degradation in some bacteria via
10 initial hydroxylating cytochrome P450s (peripheral pathway) and further *meta*-cleavage
11 of the aromatic intermediate (central pathway) [48]. The *dit* genes are usually located
12 within ICE elements [34], and we have found orthologous *dit* genes within a putative
13 ICE_{DIT} element in the genome of strain CIB (Figs. 1 and 4, Table 2). Nevertheless,
14 further work should be done to demonstrate that the predicted *dit* genes are involved in
15 abietic acid degradation in strain CIB.
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18 Steroids have been shown to generate aromatic intermediates during their
19 degradation [18]. In the genome of strain CIB we have found orthologs of the *tesEFG*
20 genes (Fig. 4A), which encode a *meta*-cleavage lower pathway [21], linked to genes
21 encoding several putative steroid dehydrogenases. Since these genes are located at
22 genomic island VI of *Azoarcus* sp. CIB (Table 2, Fig. 1), they appear to be recruited by
23 this strain and some related *Azoarcus* strains, e.g, MF63 (Table 2), as an adaptation
24 mechanism to metabolize certain steroids.
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29 *Genes for the metabolism of other carbon sources*

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31 Substrate diversity studies accomplished here (see the "Substrate diversity
32 studies" section in Materials and Methods) revealed that the major carbon sources, other
33 than aromatic compounds, for *Azoarcus* sp. CIB are monocarboxylic acids (acetate,
34 propionate, lactate, pyruvate, butyrate, 3-hydroxybutyrate, isobutyrate, valerate,
35 isovalerate), dicarboxylic acids (succinate, fumarate, malate, glutarate, adipate,
36 pimelate), and amino acids (Ala, Pro, Glu, Asp, Arg, Asn, Gln, Ile, Leu, Gly, Val, His,
37 Ser). The genes encoding all enzymes and isoenzymes of the tricarboxylic acid cycle are
38 distributed across the entire genome of strain CIB. Moreover, *Azoarcus* sp. CIB contains
39 the *aceA* and *aceB* genes encoding the isocitrate lyase and malate synthase, respectively,
40 responsible for a glyoxylate shunt that is required when growth is based on the
41 utilization of substrates, such as fatty acids or aromatic compounds, that generate acetyl-
42 CoA units, which are then channeled into anabolic pathways such as gluconeogenesis.
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46 The genome of *Azoarcus* sp. CIB appears to be devoid of a classical glycolysis
47 pathway (Embden-Meyerhoff pathway) because a *pfk* gene encoding
48 phosphofructokinase could not be identified. However, all genes needed for a
49 gluconeogenesis pathway are present in the CIB genome. Interestingly, a cluster
50 containing the *zwf* (glucose-6-P 1-dehydrogenase), *pgl* (6-phosphogluconolactonase),
51 *edd* (phosphogluconate dehydratase), and *eda* (2-dehydro-3-deoxyphosphogluconate
52 aldolase) genes that encode an Entner-Doudoroff pathway for the degradation of
53 glucose-6-phosphate, is present in the genome of strain CIB. A similar gene cluster is
54 also present in *Azoarcus* sp. KH32C and MF63 strains, but it is lacking in BH72 and
55 EbN1 strains. Although the *gnd* gene of the oxidative branch of the pentose phosphate
56 pathway appears to be absent, the genes encoding the non-oxidative branch of the
57 pentose phosphate pathway are present in the genome of strain CIB, as expected from
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1 its requirement for the biosynthesis of pentose sugars. However, despite the enzymatic
2 potential of strain CIB to degrade certain sugars (e.g., glucose, gluconate, glycerol),
3 analysis of its genome revealed the absence of genes encoding active transporters for
4 these carbon sources, which would explain why *Azoarcus* sp. CIB does not generally
5 utilize carbohydrates as growth substrates. The inability to utilize exogenous
6 carbohydrates appears to be a common feature within some strains of the *Azoarcus*
7 genus [28,38]. The fact that these *Azoarcus* strains contain genes likely to encode the
8 degradation of some sugars but they do not use such compounds when supplied as
9 carbon sources could reflect that these genes are devoted to use sugar-phosphates
10 derived of the turnover of endogenous polysaccharides from cellular envelopes or
11 storage material.

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13 Two different gene clusters, AzCIB_4593-4594 and AzCIB_0059-0062,
14 encoding putative formate dehydrogenases for the oxidation of formate to CO₂ [24]
15 have been identified in the genome of strain CIB. The role of these predicted formate
16 dehydrogenases could be to recycle the formate generated during the metabolism of
17 certain carbon sources, such as in the aerobic degradation of benzoate [19,57].
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20 *Genes coding for potential solvent and heavy metals resistance*

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24 We have identified in the genome of strain CIB several gene clusters that may
25 encode RND-type efflux systems analogous to the AcrAB/TolC-like systems of strain
26 EbN1 that were shown to be induced in the presence of aromatic solvents [62] and to
27 the TtgABC-like systems involved in aromatic hydrocarbons tolerance in *Pseudomonas*
28 strains [41] (Table S3). The AcrAB/TolC-like encoding genes are located next to the
29 gene clusters for diterpenes (*dit*) and phenylethylamine (*pea*) degradation (Table S3,
30 Fig. 4). Two additional gene clusters, one that shows similarity to a solvent-induced
31 RND-type gene cluster in *P. putida* [12], and other analogous to the *ttg2ABCDEF* genes
32 that encode an efflux pump of the ATP-binding cassette family involved in multidrug
33 resistance and toluene tolerance in *P. putida* DOT-T1E [17], were also identified in
34 *Azoarcus* sp. CIB (Table S3). The extensive repertoire of gene clusters encoding
35 putative solvent efflux pumps in *Azoarcus* sp. CIB may reflect an adaptive response of
36 this bacterium when exposed to a wide range of toxic substances that were likely
37 present in the diesel fuel-contaminated aquifer from which it was isolated [20].
38 Nevertheless, we cannot discard that some of these efflux pumps are involved in
39 extrusion of natural compounds, such as plant produced flavonoids (see below), and
40 therefore their participation in solvent tolerance will require further experimental
41 demonstration.
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47 One interesting outcome of the *Azoarcus* sp. CIB genome analysis is the
48 evidence of a high number of gene clusters encoding potential resistance to heavy
49 metals (Table S4). It is worth noting that the majority of the predicted heavy metal
50 resistance genes in *Azoarcus* sp. CIB are clustered in genomic island VII (Table 2, Fig.
51 1). The high number of putative solvent and heavy metal resistance gene clusters
52 identified in the genome of strain CIB predicts an environmentally-relevant feature of
53 this bacterium that remained unknown, and suggest promise in using this organism to
54 treat sites containing mixed wastes of aromatic solvents and metals.
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Genes for an endophytic lifestyle

Rhizosphere is a nutrient-rich microbial hotspot. To gain a competitive advantage, some of the rhizosphere bacteria penetrate plant organs and share both saprophytic and endophytic lifestyles. Recent studies have revealed that the capacity to colonize plants endophytically cannot be reduced to a few genetic traits and that different bacteria have evolved differently in their adaptation to the plant environment [36]. In this sense, it appears that plant colonization ability involves the combination of different genetic determinants, some of which are also present in non-endophytic bacteria, while other may be missing in well-characterized endophytes. So far, no unique gene cluster could be exclusively linked to the endophytic lifestyle [36]. We have searched the genome of *Azoarcus* sp. CIB to identify genes similar to those that were shown to encode functions relevant for an endophytic lifestyle in selected endophyte genomes such as that of the closely related species *Azoarcus* sp. BH72 [28].

The root surface colonization is guided by plant-released compounds, i.e., root exudates, that serve as chemoattractants, and bacterial motility is generally achieved by flagella and type IV pili (twitching motility) [7]. In *Azoarcus* sp. CIB, the genes involved in the biosynthesis and function of flagella and pili are organized in three noncontiguous gene clusters, respectively (Table S5). Although there is a conserved *che* cluster involved in the chemotaxis apparatus, an additional *cheII* cluster (Table S5), that is not conserved in most *Azoarcus* strains, is located within genomic island IX (Table 2) near to the ICE_{DIT} element (Fig. 1).

Flagella and type IV pili are also involved in bacterial adhesion to roots, which is a major feature for successful plant colonization [36]. Other frequent adhesins for root surface colonization are capsular material and exopolysaccharides. *Azoarcus* sp. CIB contains several gene clusters located within genomic islands (Table 2) distributed along the chromosome and likely devoted to the synthesis of these adhesins (Table S5, Fig. 1). Motility and the production of plant cell wall-degrading enzymes, e.g. glycoside hydrolases, might be involved in the colonization and spreading of the endophytes within the plant. Once inside plants, endophytes must have the capacity for quick adaptation to a highly different environment and they need also to overcome plant defense responses to the invasion, e.g., production of reactive oxygen species (ROS) [36]. In this sense, the genome of *Azoarcus* sp. CIB contains genes likely coding for some glycoside hydrolases and for detoxification of ROS, such as catalases, superoxide dismutases, peroxidases, hydroperoxide reductases and glutathione-S-transferases. Moreover, some of the RND-family efflux pumps that were suggested to be involved in solvent resistance (Table S3) may be also required for the export of plant toxins [8]. It is worth noting that the CIB genome contains several copies of the *phb* genes encoding proteins for the synthesis and degradation of polyhydroxybutyrate (PHB), a carbon storage compound that may enhance survival during starvation and tolerance to stress [26,54], and that could serve also as a redox regulator for the removal of growth inhibitory plant secondary metabolites [3].

Rapid adaptation of bacteria to increased osmolarity may aid plant colonization, and thus endophytes are usually provided with hyperosmotic stress response mechanisms [35]. *Azoarcus* sp. CIB contains genes orthologs to those involved in the transport and metabolism of different types of bacterial osmolytes, such as trimethylamine *N*-oxide (TMAO), polyamines and sarcosine (Table S5), located within genomic island VIII (Table 2, Fig. 1). Protein secretion systems, particularly type II, IV and VI systems, play an important role in beneficial plant-microbe interactions by triggering defense responses and by supporting colonization, nutrition and proliferation

1 of the bacteria [11,47,55]. We have found at least four different secretion systems, i.e.,
2 type I, II, IV and VI secretion systems, encoded in the genome of *Azoarcus* sp. CIB
3 (Table S5). Type VI secretion systems are frequently present in genomic islands [11]. In
4 this sense, a predicted type VI secretion system is encoded within genomic island XI of
5 *Azoarcus* sp. CIB (Table 2, Fig. 1). In plant/endophyte interactions the microorganisms
6 gain nutrients, e.g. plant-material derived aromatic compounds such as cinnamate, *p*-
7 coumarate, phenylpropionate (Fig. 4), and a protected niche, whereas the host benefits
8 from bacterial activities resulting in plant growth promotion, control of plant pathogens
9 and induction of systemic resistance [50]. Bacterial phytostimulation can be carried out
10 by the production/modulation of plant hormones, such as IAA, ethylene, etc., bio-
11 fertilization through nitrogen fixation and enhancement of the uptake of mineral
12 nutrients [36]. We have shown previously that *Azoarcus* sp. CIB fix nitrogen, produces
13 IAA, and solubilizes insoluble inorganic phosphate compounds [16]. The genes
14 predicted to encode some of these plant growth promoting functions, e.g. the *nif/rnfl*
15 gene cluster for nitrogen fixation (see above), have been detected in the genome of
16 *Azoarcus* sp. CIB (Table S5, Fig. 1).
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22 **Conclusions**

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24 The genome sequence of *Azoarcus* sp. CIB is a valuable addition to the body of
25 knowledge about a unique bacterium that combines the ability to degrade anaerobically
26 a high number of aromatic compounds [32,25] with the ability to colonize two different
27 ecological niches, i.e., soil and water as free-living bacteria, and the inner tissues of the
28 rice roots as a facultative endophyte [32]. Analysis of the genome of strain CIB strongly
29 suggests that horizontal gene transfer and mobile genetic elements played a major role
30 as a mechanism of adaptation of this bacterium to its different lifestyles. Comparative
31 genomics among the five sequenced *Azoarcus* strains available so far provides a pan-
32 genome that confirms the global metabolic flexibility of this bacterial genus, improves
33 the characterization of the core genome and the phylogenetic relationships among these
34 bacteria, and suggests that the phylogeny of the *Azoarcus* genus should be revisited in
35 the near future. Unraveling the complex history and role of mobile genetic elements in
36 the diversification and evolution of the *Azoarcus* species will require further
37 investigation.
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41 Analyses of the *Azoarcus* sp. CIB genome confirmed that this strain can be
42 regarded as a degradation specialist feeding on compounds that range from plant
43 exudates, e.g., dicarboxylic acids, to naturally as well as anthropogenic released toxic
44 aromatic compounds, e.g., toluene and *m*-xylene. The potential genetic determinants
45 that encode the peripheral and central pathways for the aerobic and anaerobic
46 degradation of aromatic compounds have been identified. Moreover, these analyses
47 expanded the catabolic potential of strain CIB towards common natural compounds,
48 such as certain diterpenes and probably some steroids, that were not anticipated as
49 carbon sources within the *Azoarcus* genus.
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52 The presence of a high number of putative solvent efflux pumps and heavy metal
53 resistance gene clusters in the genome of *Azoarcus* sp. CIB revealed two potential
54 environmentally-relevant features of this bacterium that remained unknown and that
55 deserve further research. The combination of a wide metabolic versatility with stress
56 resistance properties suggest promise in using *Azoarcus* sp. CIB to treat sites containing
57 mixed wastes of aromatic solvents and metals, and as a suitable biocatalyst in defined
58 communities of degradation specialists.
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1 Genome mining revealed several gene clusters likely involved in the endophytic
2 lifestyle of *Azoarcus* sp. CIB, opening the door to the molecular characterization of
3 some plant growth promoting traits [16] which makes this endophyte as a potential
4 candidate for phytostimulation, biofertilization and biocontrol in a more sustainable
5 agricultural practice [50]. Since *Azoarcus* sp. CIB is able to degrade toxic aromatic
6 compounds and resist metals, the use of this bacterium in association with plants could
7 offer an efficient, economic and sustainable phytoremediation technology [61].

8 This work paves the way for a systems biology-based understanding of the
9 abilities of *Azoarcus* sp. CIB to integrate aerobic and anaerobic metabolism of aromatic
10 compounds, tolerate stress conditions, and interact with plants as an endophyte. This
11 will ultimately lead to an increased understanding of the adaptation processes of strain
12 CIB to its different lifestyles, and will allow to elucidate further the role of the
13 individual gene clusters described in this work in the proposed target functions.
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24
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Figure legends

Fig. 1. Distribution of some mobile genetic elements, toxin-antitoxin systems (TASs) and regions of unusual GC content in the *Azoarcus* sp. CIB genome. The AzCIB_xxxx locus number in the annotated CIB genome is detailed in brackets. The color code for transposases is: black, IS4-family transposases without paralogs; light yellow and orange, IS4-family transposases with paralogs; dark yellow, IS3-family transposases; dark blue, IS91-family transposases; light blue, IS30-family transposases; light green, IS21-family transposases; dark green, IS5-family transposases; brown, ISL3-family transposase; rose, IS110-family transposases; grey, IS66-family transposases; white, IS1595-family transposase; red, IS200/IS605-family transposase (Y1 family); violet, other transposases. The 14 predicted TASs are numbered. Abbreviations used are detailed in Supplementary data.

Fig. 2. Venn diagram representing the core genome and pan genome of the five sequenced *Azoarcus* strains. The core and pan genome was calculated with the EDGAR program [6] by iterative pairwise comparison of all genomes taking as reference genome that of strain CIB. The Venn diagram shows the number of reciprocal best hits between a subset of genomes. Therefore, the number of exclusive genes in each genome is always higher than the number of singletons (a gene without any hit against any other genome) for each strain.

Fig. 3. (A) Core genome tree generated using EDGAR showing the phylogenetic positions of the five sequenced *Azoarcus* strains. The phylogenetic tree was constructed based on the nucleotide sequences of the 1701 genes that constitute the *Azoarcus* core genome. The tree was visualized by using MEGA 6 [51]. (B) Average amino acid identities (AAI, in %) calculated from the core genome data set obtained by comparing the five sequenced *Azoarcus* strains. *Thauera aminoaromatica* MZ1T strain was used as reference strain from a closely related bacterial genus.

Fig. 4. Pathways for the catabolism of aromatic compounds in *Azoarcus* sp. CIB. (A) The location of genes and genes clusters predicted to encode the aromatic catabolic pathways is indicated (plain numbers) on the complete *Azoarcus* sp. CIB genome. The AzCIB_xxxx locus number in the annotated CIB genome is detailed in brackets. The ICE_{DIT} and ICE_{XTD} elements are represented by open and filled bars, respectively. Genes responsible for anaerobic or aerobic pathways are shown in blue or red, respectively. Genes encoding central pathways are indicated in bold and italics; genes encoding peripheral pathways that funnel to central routes are indicated in plain text. Genes names and their predicted functions are detailed in Supplementary data. (B) Scheme of the predicted peripheral and central pathways for the anaerobic (in blue) or aerobic (in red) catabolism of aromatic compounds in *Azoarcus* sp. CIB. Central pathways are shown as thick arrows. Peripheral pathways are shown as thin arrows with continuous (represent one biochemical step) or discontinuous (represent several biochemical steps) lines. The names of the genes are indicated in panel A. The *hbcL*, *mbdA*, *bzdA*, *paaK*, and *bclA* genes are those encoding the corresponding aromatic acid

CoA ligases within the *hbd*, *mbd*, *bzd*, *paa* and *box* clusters, respectively. The names of the compounds are detailed in Supplementary data.

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1 **Table 1**

2 Genome features of *Azoarcus* sp. strain CIB in comparison to those of strains EbN1, BH72,
3 KH32C, and MF63.
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Genome features	CIB	EbN1	BH72	KH32C	MF63
Total size (bp)*	5,257,030	4,727,255	4,376,040	5,818,755	5,925,983
GC%	65.8	65.1	67.9	65.1	66.0
Protein	4,739	4,603	3,992	5,188	5,432
rRNA	4	4	4	5	4(?)
tRNA	57	58	56	64	48
Plasmids	0	2	0	1	ND

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24 * Total genome size, including plasmids.
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Table 2

Some genomic islands in *Azoarcus* sp. CIB and presence of ortholog genes in the genome of strains EbN1, BH72, KH32C, and MF63.

Genome island	Chromosomal location	Predicted function	<i>Azoarcus</i> genomes that contain ortholog genes
I	145 kb-261 kb	Unknown	MF63
II	589 kb-622 kb	Lipopolysaccharide synthesis	EbN1, BH72, KH32C, MF63
III	768 kb-784 kb	Nitrogen fixation	BH72, KH32C, MF63
IV	923 kb-941 kb	Exopolysaccharide synthesis	KH32C, MF63
V	1269 kb-1300 kb	Unknown	None
VI	1509 kb-1758 kb	Steroids metabolism	MF63
VII	1834 kb-2070 kb	Metals resistance	MF63
VIII	2326 kb-2394 kb	Osmotic stress resistance	KH32C, MF63
IX	2608 kb-2621 kb	Chemotaxis	MF63
X (ICE _{DIT}) ^a	2636 kb-2925 kb	Diterpenes degradation	MF63 (lacks the ICE _{DIT} element)
XI	3620 kb-3635 kb	Type VI protein secretion	KH32C, MF63
XII	4083 kb- 4118 kb	Exopolysaccharide synthesis	KH32C
XIII	4262 kb-4292 kb	Capsular material synthesis	None
XIV(ICE _{XTD}) ^a	4894 kb-5067 kb	Aromatic hydrocarbons degradation	EbN1, MF63 (lack the ICE _{XTD} element)
XV	5164 kb-5196 kb	Phage tail-like bacteriocin	KH32C, MF63

^a ICE, integrative and conjugative element.

Figure 1
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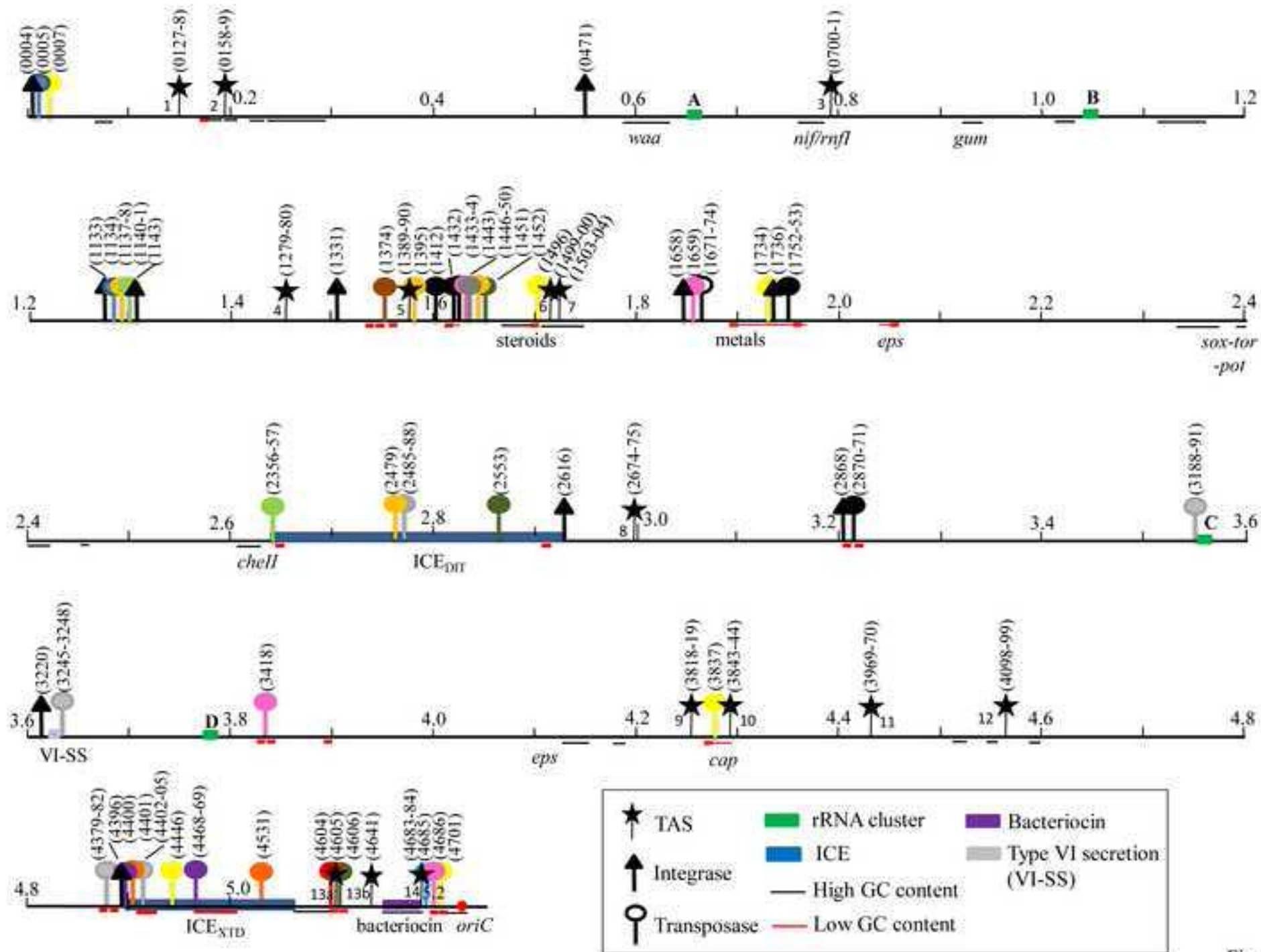


Fig. 1

Figure 2
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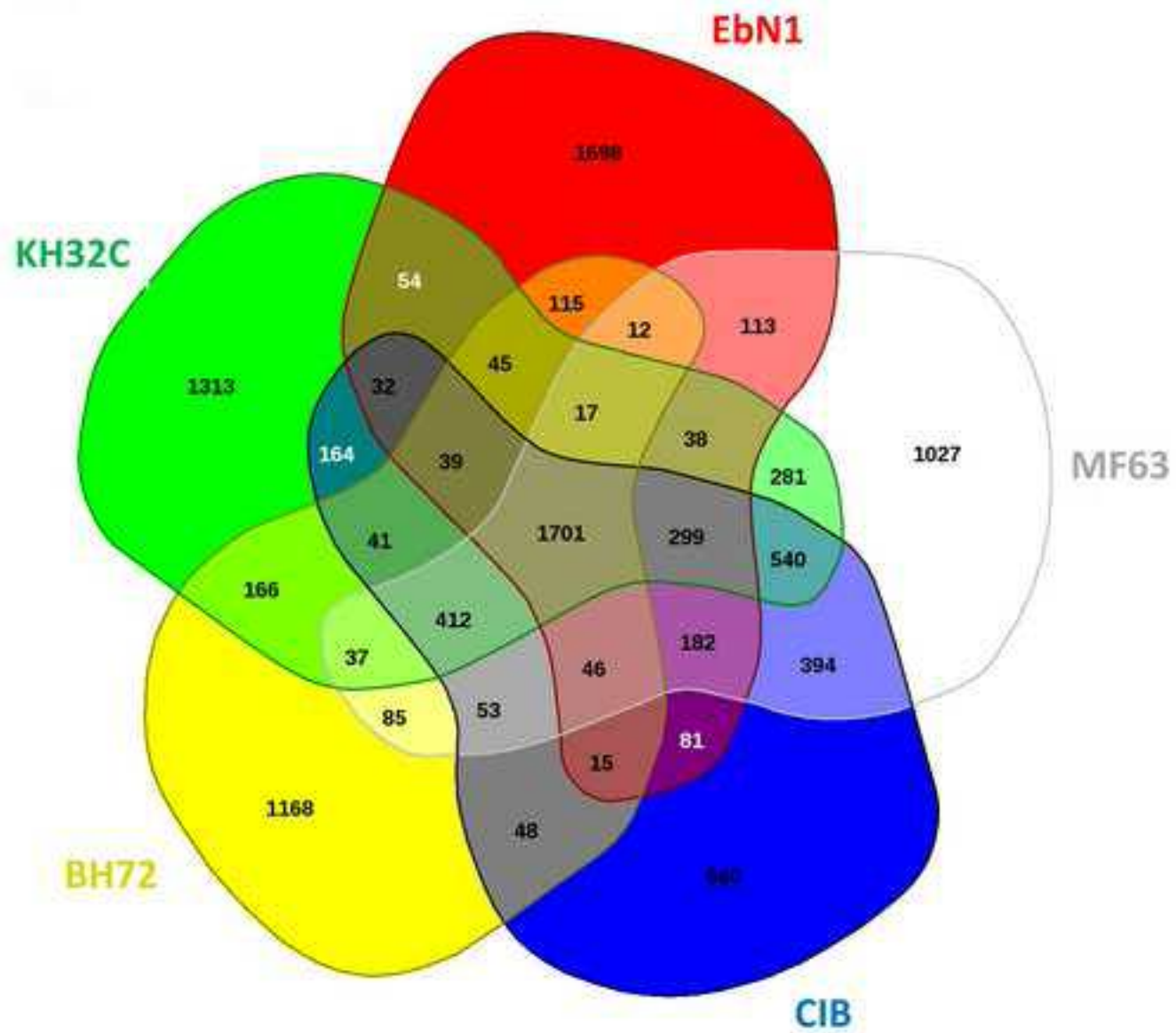
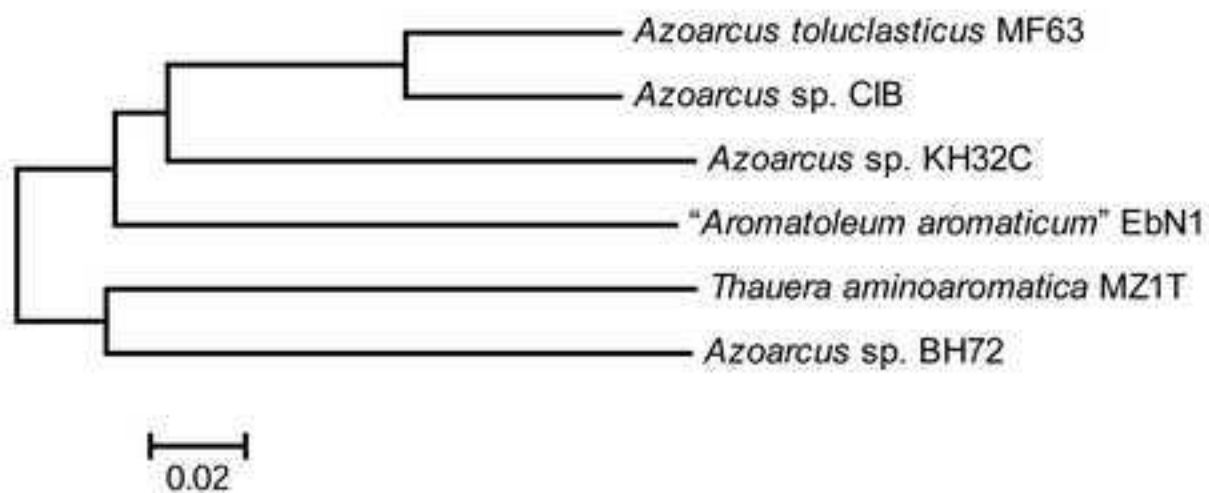


Fig. 2

Figure 3
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A



B

<i>Azoarcus toluclasticus</i> MF63	76.06	77.69	83.27	85.65	95.14	100
<i>Azoarcus</i> sp. C1B	75.84	77.61	83.31	85.63	100	
<i>Azoarcus</i> sp. KH32C	75.55	77.19	82.51	100		
" <i>Aromatoleum aromaticum</i> " EbN1	75.5	77.49	100			
<i>Azoarcus</i> sp. BH72	78.46	100				
<i>Thauera aminoaromatica</i> MZ1T	100					

Fig. 3

Figure 4
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