Title: Genomic characterization of the virulence-associated pyomelanin biosynthetic pathway in pigment-producer strains from the pandemic *Acinetobacter baumannii* IC-5.

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BACKGROUND *Acinetobacter baumannii* outbreaks have been associated with pandemic International Clones (ICs), however the virulence factors involved with their pathogenicity are sparsely understood.

OBJECTIVES This study aimed to characterize the reddish-brown pigment produced by *A. baumannii* strains, and to determine its biosynthetic pathway by genomic approaches.

METHODS Pigment characterization was conducted by phenotypic tests in different growth conditions. The clonal relationship among *A. baumannii* was obtained by PFGE and MLST and antimicrobial susceptibility test was performed by Disc-Diffusion method. The genome of one representative strain was obtained for characterization of genes involved with pigment production.

FINDINGS The virulence-associated pyomelanin was the pigment produced by *A. baumannii*. These strains were extensively drug resistant and belonged to the IC-5/ST79. Genomic approaches revealed that the pyomelanin biosynthetic pathway in *A. baumannii* presented a particular architecture concerning the peripheral (*tyrB*, *phhB* and *hpd*) and central (*hmgB*, *hmgC* and *hmgR*) metabolic pathway genes. The identification of a distant HmgA homologue, probably without dioxygenase activity, could explain pyomelanin production. Virulence determinants involved with adherence (*csuA/BABCDE* and a T5bSS-carrying genomic island), and iron uptake (*basABCDEFGHIJ*, *bauABCDEF* and *barAB)* were also characterized.

MAIN CLONCLUSION The pyomelanin production together with other virulence determinants could play a role in *A. baumannii* pathogenicity.

Keywords: International clone – virulence - pyomelanin pigment – adherence - extensively drug resistance – persistence

Acinetobacter baumannii is one of the most relevant pathogens associated with nosocomial infections that presents the long-term ability to survive on inanimate surfaces, contributing to national and international clonal dissemination.[1] The *A. baumannii* outbreaks have been associated with high-risk pandemic lineages, named International Clones (ICs), characterized by a high capacity to persist in clinical environments and by presenting a broad antimicrobial resistance profile.[2,3] However, in spite of *A. baumannii* association with nosocomial and persistent infections, the role of virulence factors in its pathogenesis remains largely obscure. This virulence has been associated with features that enhance its persistence, such as increased adherence, resistance to dissection, biofilm formation, production of capsule and iron uptake.[4-6]

In bacteria, the production of pigments, as melanins, have been linked with virulence and pathogenicity. Melanins are a black-brown and yellow-red pigments derived from the oxidation of different phenolic compounds.[7] Depending on the biosynthesis pathway, melanin may be given a different designation, such as pyomelanin, which is a reddish-brown pigment resulted from tyrosine (Tyr) or phenylalanine (Phe) through the accumulation of homogentisic acid (HGA).[8] This pigment provides protection against oxidative stress and contribute to invasiveness and persistence by enhancing bacterial surface attachment and biofilm formation, extracellular electron transfer, resistance to heavy-metals and iron reduction/acquisition, induction of virulence factor expression, contributing to the adaptive response to environmental stress.[9,10] The pyomelanin production results from a defect in the catabolism pathway. *Pseudomonas putida* metabolizes Phe and Tyr through a peripheral pathway, regulated by the σ^{54}-dependent transcriptional activator PhhR, involving hydroxylation of Phe to Tyr by PhhAB, conversion of Tyr into 4-hydroxyphenylpyruvate by TyrB, and formation of HGA by Hpd as the central

intermediate. HGA is then catabolized by a central catabolic pathway that involves the homogentisate dioxygenase (HmgA), fumarylacetoacetate hydrolase (HmgB), and maleylacetoacetate isomerase (HmgC), finally yielding fumarate and acetoacetate.[11] Mutations or deletions that result in loss of HmgA function, as well as overexpression of *hmgR*, a *hmgA* repressor from the Tet^R family, lead to an accumulation of HGA.[12,13] The accumulated HGA is then secreted from the cell via the HatABCDE ABC transporter, where it auto-oxidizes, and self-polymerizes to form pyomelanin.[14] The production of this pigment is quite common in species such as *Legionella, Vibrio cholera* and *Pseudomonas sp.*.[9-13] However, pyomelanin production in *A. baumannii* was only reported in a lineage causing nosocomial infections in Rio de Janeiro city, Brazil, in 2010.[15]

This study reports the occurrence of persistent *A. baumannii* strains producing a brown diffusible pigment resembling the pyomelanin, which caused an outbreak in a hospital of the Amazon Basin, Brazil. Based on whole genome analyses of a representative strain, we characterized the biosynthetic pathway involved with the production of this pigment, which could contribute to the virulence of this *A. baumannii* lineage.

MATERIAL AND METHODS

Clinical data, bacterial strains and antimicrobial susceptibility test - From October, 2016 to April, 2018, 12 *A. baumannii* producing a brown diffusible pigment were recovered from inpatients hospitalized in different wards of the General Hospital of Roraima (HGR), placed in Boa Vista, a city embedded in the Amazon Basin, Brazil (TABLE). Species identification was determined by the automated VITEK2, and confirmed to be *A. baumannii* by sequencing the 16S rRNA and the bla_{OXA-51} genes.

The antimicrobial susceptibility test was determined by the disc-diffusion method, according to CLSI guidelines,[16] for the following antibiotics: gentamicin, amikacin, tobramycin, imipenem, meropenem, doripenem, ciprofloxacin, ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanic acid, cefotaxime, ceftazidime, cefepime, trimethoprim/sulphamethoxazole, tetracycline and minocycline. The MIC of polymyxin B was assessed by the broth microdilution with antibiotic concentrations ranged from 0.1 μg/ml to 64 μg/ml. The current definition criteria for classifying *A. baumannii* antimicrobial resistance was applied.[17]

Phenotypic characterization of brownish pigment produced by A. baumannii strains - The strains were grown overnight on Mueller-Hinton (MH) and trypticase soy agar (TSA) media plates at different temperatures (28ºC, 35ºC and 40ºC) to verify the influence on pigment production. To investigate whether the pigment is the pyomelanin resulted from the tyrosine metabolism, the 12 pigment-producing *A. baumannii* strains were grown in a minimal medium (T-Medium),[18] with the tyrosine and glutamate as the sole carbon sources. The pyomelanin-producing *A. baumannii* 456MDp,[15] kindly provided by Dr. Beatriz M. Moreira, and the *A. baumannii* ATCC 19606 were also included in this test as positive and negative controls, respectively.

An additional test was performed to determine which tyrosine metabolic pathway was involved with the brown pigment production. Therefore, the effect of sulcotrione [2-(2-chloro-4-methane sulfonylbenzoyl)-1,3-cyclohexanedione)], an inhibitor of tyrosine metabolism via homogentisic acid,[19] was evaluated by growing the isolates in the T-medium in the presence of different concentrations (2.5, 10, 15 and 20 mM) of sulcotrione.

126 *Determination of genetic relatedness of A. baumannii strains* - The genetic relationship
127 among the 12 pigment-producing *A. baumannii* strains and between these strains and
128 the pyomelanin-producing *A. baumannii* 456MDp, previously identified in a hospital
129 from Rio de Janeiro,[15] were assessed by PFGE and MLST using the Pasteur and
130 Oxford schemes (https://pubmlst.org/abaumannii/) available in the *A. baumannii* MLST
131 website.
132
133 *Whole genome sequencing and genome annotation* - The genome sequence of one
134 representative pigment-producing strain (AB4353) were obtained with the Illumina
135 HiSeq 2500 sequencer using Nextera XT paired-end run with a ˜500-bp insert library at
136 the High-Throughput Sequencing Platform of the Oswaldo Cruz Foundation (Fiocruz,
137 Rio de Janeiro, Brazil). The quality of the reads was assessed with FASTQC and *de*
138 *novo* assembling was performed with the SPAdes 3.5 assembler with default settings.
139 Gene prediction and annotation were performed with RAST tool and Prokka software
140 (https://github.com/tseemann/prokka). The resistome was assessed with the
141 Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/).
142 The mobilome and virulome were assessed with IslandViewer4
143 (https://www.pathogenomics.sfu.ca/islandviewer/) and VRprofile 2.0 (https://bioinfo-
144 mml.sjtu.edu.cn/VRprofile/) web servers, respectively. AB4353 genome sequence has
145 been submitted to GenBank under accession no. JAAXKU000000000.1.
146

147 **RESULTS AND DISCUSSION**

148
149 *Characterization of brownish-producer A. baumannii strains* - The 12 clinical *A.*
150 *baumannii* strains producing a brown diffusible pigment were phenotypically and

151 genotypically characterized. All of them presented the extensively-drug resistant (XDR)
152 phenotype, since they were susceptible only to polimixin B and tetracyclines.

153 All strains were able to produce the pigment on MH medium at all tested
154 temperatures, with a more prominent production at higher temperatures (35ºC and 40ºC)
155 (Fig. 1A, data shown for AB4353, AB1077, AB1113, AB41-RR4), as previously
156 demonstrated.[15,20] It was verified that production of pigment at higher temperatures
157 was due to the induction of *melA* (*hpd*), which is responsible for the HGA synthesis,[20]
158 suggesting the role of this mechanism in the adaptive response to environmental stress.
159 On the other hand, no pigment was observed on TSA medium.

160 Phenotypic tests to verify whether the brown pigment resulted from the tyrosine
161 catabolic pathway revealed that, after 20h of incubation on T-medium in different
162 temperatures, a brown diffusible pigment was observed in the 12 *A. baumanniii* and in
163 the positive control 456MDp strain (Fig. 1B, data shown for AB4353, AB77 and
164 456MDp), while no pigment production was observed on the negative control ATCC
165 19606 (data not shown). Moreover, a significant reduction in the brown pigment
166 production occurred on T-medium plus 2.5 µM of sulcotrione (Fig. 1B). Considering
167 that this substance is an inhibitor of tyrosine metabolism via HGA pathway, and that
168 pyomelanin production results from its accumulation and efflux, it could be inferred that
169 this brownish pigment corresponded to pyomelanin.
170

171 *Genetic relatedness and epidemiology of pyomelanin-producer A. baumannii* - The PFGE
172 revealed that the 12 XDR pyomelanin-producing *A. baumannii* strains were clonally related
173 (TABLE), however, no genetic relationship was observed between these strains and the
174 pyomelanogenic *A. baumannii* 456 MDp strain recovered from a hospital in Rio de Janeiro
175 in 2010.[15] All 12 strains belonged to $ST79^{PAS}$/$ST758^{OXF}$, which corresponds to the high-

risk pandemic International Clone V (IC-5), while the 456MDp strain belonged to ST1079^{PAS}/ST1483^{OXF} already identified in China in 2015 (MLST metadata). The IC-5 is prevalent in clinical settings spread in Brazil and South America,[21] however, the pigment production has never been highlighted as a phenotypic trait of this IC neither in Brazil nor in other continents. Interestingly, this lineage has persisted in HGR for more than one year (19 months), which could be resulted from an increased adaptive fitness.

The resistome of AB4353 - Resistome prediction analyses of AB4353 revealed the presence of several genes, conferring resistance to aminoglycosides (*aac(6')-Ian*, *aac(3')-IIe*, *aph(3'')-Ib*, *aph(6')-Id*), chloramphenicol (*cmlA*), sulfonamide (*sul1*), β-lactams (bla_{TEM-1b} and bla_{OXA-65}) and carbapenems (bla_{OXA-23}), corroborating the observed XDR phenotype.

Virulome of pyomelanin-producer AB4353 strain: adherence, iron uptake and desiccation tolerance - Bacterial adherence constitutes an essential step in the colonization process. *In silico* analysis of AB4353 genome revealed the presence of a 18 kb adherence-related genomic island previously identified in AbH120-A2, an *A. baumannii* strain with a remarkable adherence ability responsible for a large nosocomial outbreak in Spain from 2006 to 2008.[22] This island carried the Type Vb secretion system from the two-partner System (TPS) family composed by TpsA (AbFhaB) and TpsB (AbFhaC), a large exoprotein involved with Heme utilization and adhesion and its translocator channel, respectively. This adhesion-related secretion system had already been found in other Gram-negative bacteria, and it is considered one of the main virulence factors in *Bordetella pertussis*.[23] Interestingly, the AbH120-A2 (2006-2008) from Spain and AB4353 (2017) from Brazil belongs to the IC-5 (ST79), demonstrating

the increased adaptive fitness and the remarkable spread potential of this lineage. Such adaptation could be due to the presence of this adhesion-related island, among other factors, which has been probably contributing to IC-5 persistence in clinical settings worldwide for, at least, 10 years. Additionally, other determinants associated with biofilm formation and adherence phenotypes were also identified in AB4353, such as the biofilm-associated protein (Bap) and the CsuA/BABCDE usher-chaperone system.[24-26]

The iron uptake capacity has been considered an important component for bacterial growth and survival under iron-limiting conditions found in host environment, also contributing to pathogenicity. The AB4353 harbour the siderophore Acinetobactin operon identical to that found in the ATCC 19606^T, composed by *basABCDEFGHIJ*, *bauABCDEF and barAB* genes, involved with biosynthesis, utilization and siderophore release, respectively.[27]

Desiccation tolerance contributes to the remarkable persistence character of *A. baumannii*, allowing it to become a successful pathogen in the nosocomial environment. The two-component System BfmRS is directly involved with the production of the desiccation resistance phenotype in this species.[28] Two residues in BfmR, Leu230 and Thr85, are crucial to the BfmR activity and the control of stress responses, which protect *A. baumannii* cells during desiccation. The deduced BfmR from AB4353 presented the canonical residues and is identical to that of profoundly desiccation-tolerant strains,[28] indicating that AB4353 may have this desiccation tolerance phenotype. In fact, as aforementioned, this strain has persisted in HGR clinical settings for, at least, 19 months. Moreover, it has been shown that the copy number of *umuD* and *umuC* error-prone DNA polymerase V genes may directly contribute to desiccation-induced mutagenesis.[29] AB4353 presented one copy of *umuD* and three copies of

226 *umuC*, which may be contributing to increase the mutagenesis rates involved with
227 desiccation-tolerant phenotype.
228
229 *Genomic characterization of the pyomelanin biosynthetic pathway* - Pyomelanin
230 biosynthetic pathway is well known in *Pseudomonas* species.[13,14,18,20] However,
231 although the pyomelanin production had already been demonstrated in *A. baumannii*,[15]
232 its biosynthesis remains to be characterized in this species. Thus, we performed
233 comparative genomic analysis to identify and characterize the genes involved with
234 pyomelanin production in AB4353. Homolog genes of *hmgR*, *hmgB* and *hmgC*,
235 involved with pyomelanin central catabolic pathway, were characterized in AB4353,
236 sharing 29%, 45% and 46% deduced amino acid identity with those from *P. putida*,
237 respectively. A homologue of *aroP2* gene, which encodes an aromatic amino acid
238 permease, was found contiguous to the putative *hmgB* in AB4353 (Fig. 2), with a gene
239 arrangement similar to that of *P. putida* KT2440.[11]
240 The HmgA is a ring-cleaving dioxygenase from the Dioxygenase Superfamily.
241 Although no *hmgA* homologue has been identified in AB4353, a putative gene whose
242 deduced product presented the type I ring-cleaving dioxygenase conserved domain,
243 which is related to the main function of HmgA, was found between *hmgC* and *hmgR* in
244 AB4353 (Fig. 2). Therefore, it suggested the presence of a distant homologue of *hmgA*
245 with no dioxygenase activity in this strain.
246 The *in silico* analyses revealed that AB4353 harboured the *phhR*, *hpd* and *tyrB*
247 genes from the peripheral pathway (Fig. 2), which presented 42%, 67% and 45% amino
248 acid identity with those from *P. putida*, respectively. As found for some other genera,
249 the *hpd* and *tyrB* were not linked to the *phh* operon in AB4353, as found for *P.*
250 *putida*.[11] In fact, the *phhAB* were absent in AB4353, and the *hpd* was associated with

the *hmg* genes (Fig. 2), as observed in *Pseudomonas syringae*, *Xanthomonas axonopodis*, *Caulobacter crescentus*, *Bradyrhizobium japonicum*, *Mesorhizobium loti*, and *Sinorhizobium meliloti*.[11] Considering that the conversion of phenylalanine in tyrosine is mediated by *phhAB,* and that these genes are absent in AB4353, it can be assumed that a pathway other than hydroxylation of phenylalanine is probably involved in the tyrosine biosynthesis as demonstrated elsewhere.[11]

Comparison of the peripheral and central pathways of *Pseudomonas* species and other genera demonstrated a high heterogeneity in gene synteny.[11] In fact, AB4353 displayed a new gene organization concerning both those involved with peripheral and central pathways. Interestingly, a conserved synteny of pyomelanin pathway genes was observed among AB4353 and two other genomes (AB120-02 and AB421) also from IC-5 (ST79) recovered from outbreaks in Spain in 2006-2008 and 2010.[22,30] The unique difference is that in AB421 the *hmgC* was separated from the putative ring-cleaving dioxygenase by a 3.6 kb segment. As aforementioned, the pyomelanin formation depends on the export of the accumulated HGA. AB4353 harboured the entire *hat* ABC transporter gene cluster, sharing 57% (HatA), 69% (HatB), 35% (HatC), 31% (HatD) deduced amino acid identity with those from *P. aeruginosa* UCBPP-PA14.[14]

Therefore, i) the presence of the peripheral pathway genes (*phhR*, *tyrB* and *hpd*) responsible for HGA formation from tyrosine metabolism; ii) the presence of a distant *hmgA* homologue, which is probably not functional, resulting in the HGA cytoplasmic accumulation; and iii) the presence of *hatABCDE* ABC transporter, which pumps HGA, allowing it to self-polymerize into pyomelanin out of the cell; indicate that AB4353 presents the minimum requirements for pyomelanin biosynthesis, and that its production involves a pathway similar to that described in *Pseudomonas* species.[11,12]

CONCLUSIONS

The production of pyomelanin, a pigment associated with virulence in bacteria, by *A. baumannii* strains belonging to the pandemic IC-5, and the existence of a set of genes related to increased adherence and iron uptake, could play a major role in the virulence and persistence of this pandemic lineage.

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AUTHOR CONTRIBUTIONS

EF – Conceptualization and design of the study, performed the experiments, analyzed and interpreted the data, wrote, reviewed and edited the manuscript; RC – collected the bacterial strains; FF and SM – performed the experiments; ACV - Conceptualization and design of the study, scientific supervision, funding acquisition, revision, edition and final approve the manuscript. All authors have read and agreed to the published version of the manuscript.

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413 **TABLE**

414 Clinical and genotypic features of the XDR pyomelanogenic *A. baumannii* strains

	Isolation date	PFGE	MLST (IC)	Ward	Clinical specimen
AB4353	Oct/21/16	A	ST79 (IC-5)	ICU	Catheter tip
AB77	Jan/05/17	A	ST79 (IC-5)	others hospital wards	Wound secretion
AB2299	Feb/12/17	A	ST79 (IC-5)	Emergency	Bronchial aspirate
AB1077	Mar/08/17	A	ST79 (IC-5)	ICU	Tracheal secretion
AB1113	Mar/08/17	A	ST79 (IC-5)	ICU	CRL
AB81	Oct/02/17	A1	ST79 (IC-5)	ICU	Tracheal secretion
AB04-RR5	Jan/01/18	A1	ST79 (IC-5)	ICU	Blood
AB28-RR5	Jan/17/18	A	ST79 (IC-5)	ICU	Tracheal secretion
AB41-RR4	Jan/19/18	A	ST79 (IC-5)	ICU	Tracheal secretion
AB51-RR5	Jan/26/18	A	ST79 (IC-5)	ICU	Tracheal secretion
AB04-RR6	Apr/25/18	A	ST79 (IC-5)	ICU	Catheter tip
AB05-RR6	Apr/29/18	A	ST79 (IC-5)	others hospital wards	Catheter tip

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416 CRL, Cephalorachidian liquid

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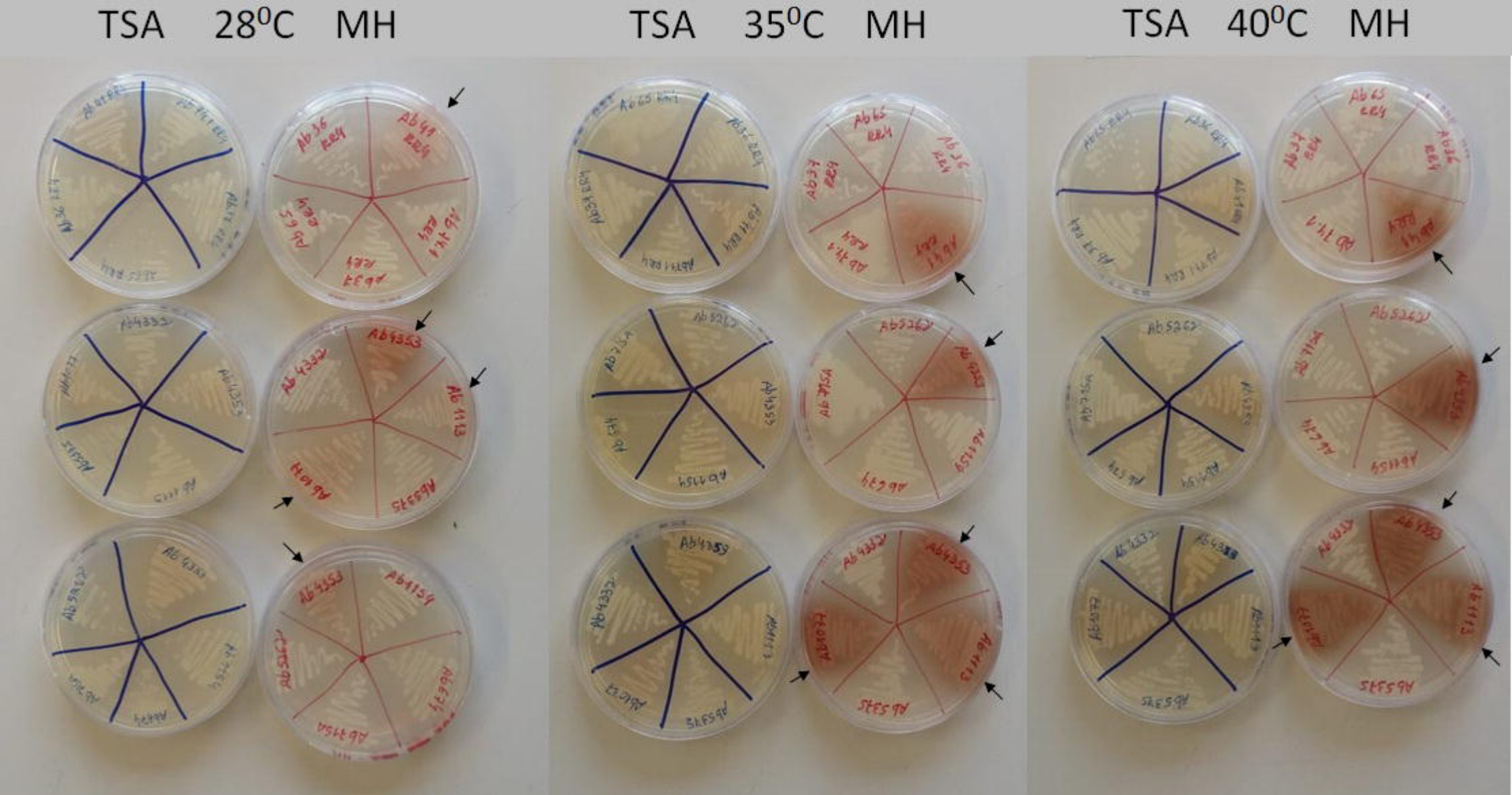
Fig. 1: Pyomelanin production by the XDR *A. baumannii* strains. (A) The conditions and temperatures used in the test are shown. Arrows indicate the pyomelanogenic *A. baumannii* from this study chosen as representative strains for these tests (AB4353, AB1077, AB1113, AB41-RR4). Other *A. baumannii* strains were used as negative controls; **(B)** Production of pyomelanin at minimal T-medium in the presence and in the absence of sulcotrione inhibitor at different temperatures. The image shows the pigment production by two pyomelanogenic *A. baumannii* representative strains of this study (AB4353 and AB77) and by the 456MDp strain used as positive control.

452 **Fig. 2: Gene organization of the pyomelanin biosynthetic pathway in *A. baumannii***

453 **AB4353**. Genes are represented by arrows and the central and peripheral pathways

454 genes are highlighted with different patterns.

455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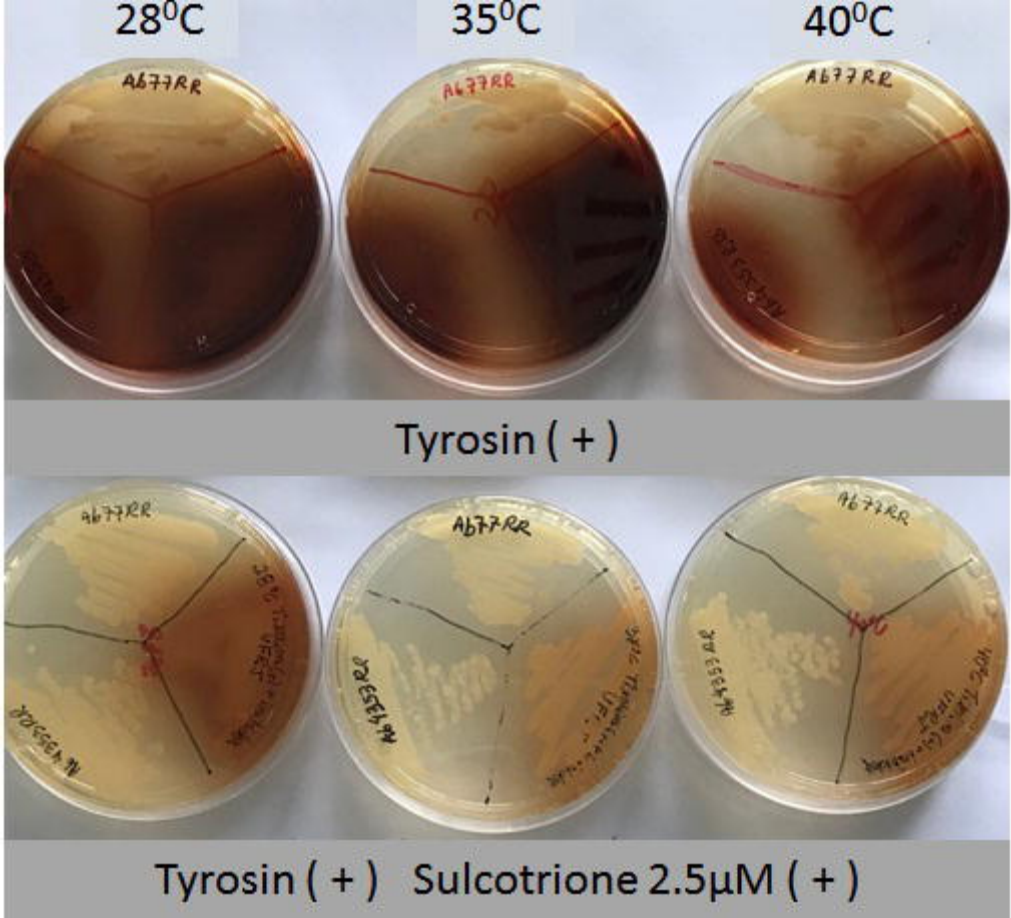
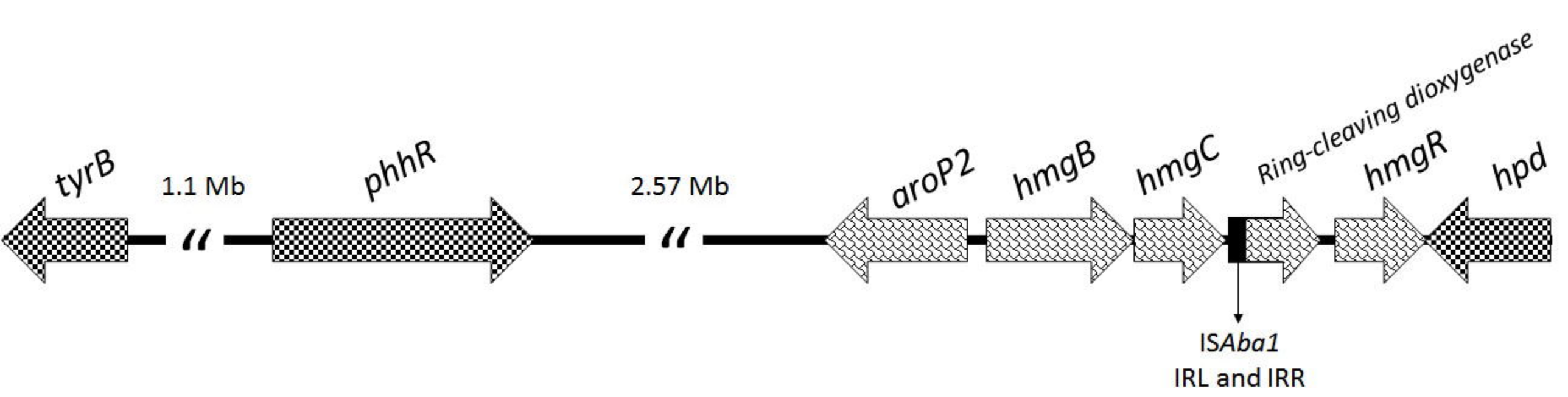
TSA 35°C MH
TSA 40°C MH


28⁰C
35⁰C
40⁰C
Tyrosin (+)
Tyrosin (+) Sulcotrione 2.5µM (+)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phhR
2.57 Mb
aroP2
hmgB
hmgC
Ring-cleaving dioxygenase
hmgR
hpd
ISAba1
IRL and IRR
PERIPHERAL PATHWAY
CENTRAL PATHWAY