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Biological Markers of *Pseudomonas aeruginosa* Epidemic High-Risk Clones

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A limited number of *Pseudomonas aeruginosa* genotypes (mainly ST-111, ST-175, and ST-235), known as high-risk clones, are responsible for epidemics of nosocomial infections by multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains worldwide. We explored the potential biological parameters that may explain the success of these clones. A total of 20 isolates from each of 4 resistance groups (XDR, MDR, ModR [resistant to 1 or 2 classes], and MultiS [susceptible to all antipseudomonals]), recovered from a multicenter study of *P. aeruginosa* bloodstream infections performed in 10 Spanish hospitals, were analyzed. A further set of 20 XDR isolates belonging to epidemic high-risk clones (ST-175 [*n* = 6], ST-111 [*n* = 7], and ST-235 [*n* = 7]) recovered from different geographical locations was also studied. When unknown, genotypes were documented through multilocus sequence typing. The biological parameters evaluated included twitching, swimming, and swarming motility, biofilm formation, production of pyoverdine and pyocyanin, spontaneous mutant frequencies, and the *in vitro* competition index (CI) obtained with a flow cytometry assay. All 20 (100%) XDR, 8 (40%) MDR, and 1 (5%) ModR bloodstream isolate from the multicenter study belonged to high-risk clones. No significant differences were observed between clonally diverse ModR and MultiS isolates for any of the parameters. In contrast, MDR/XDR high-risk clones showed significantly increased biofilm formation and mutant frequencies but significantly reduced motility (twitching, swimming, and swarming), production of pyoverdine and pyocyanin, and fitness. The defined biological markers of high-risk clones, which resemble those resulting from adaptation to chronic infections, could be useful for the design of specific treatment and infection control strategies.

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (1–3). This growing threat results from the interplay of the extraordinary capacity of this pathogen for developing resistance to nearly all available antibiotics by the selection of mutations in chromosomal genes and from the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases (metallo-β-lactamases [MBLs]) or extended-spectrum β-lactamases (ESBLs), frequently cotransferred with genes encoding aminoglycoside-modifying enzymes (4). Over the last decade, multiple reports have warned about the epidemic dissemination of XDR/MDR strains in multiple hospitals (5–10). Even more concerning are recent reports which have provided evidence of the existence of MDR/XDR clones of *P. aeruginosa* disseminated in multiple institutions worldwide, denominated epidemic high-risk clones (11). Among them, ST-111, ST-175, and ST-235 are the most widespread (12–18). Moreover, in a recent multicenter study of *P. aeruginosa* bloodstream infections, we showed that all XDR isolates (10.5% of all isolates) and some MDR isolates belonged to the epidemic high-risk clones, which were not detected among susceptible isolates (19); the specific genetic resistance markers of these clones, which may include multiple combinations of chromosomal mutations and/or horizontally acquired resistance elements, were also described in detail for the first time (19).

The global success of bacterial pathogens is expected to be determined by a complex interplay between pathogenicity, epidemicity, and antibiotic resistance (20). The fitness cost of antibiotic resistance mechanisms (21), the existence of regulatory networks interconnecting resistance and virulence (22, 23), and natural genetic engineering linking antibiotic resistance determinants and clonal success through genetic capitalism (24) are thought to be the main elements of this intricate equation (25). While the impacts on virulence and fitness of several individual antibiotic resistance mechanisms of *P. aeruginosa* have been evaluated (26–28), the specific adaptive traits that may explain the global success of epidemic high-risk clones remains unexplored. Since understanding the reasons for the success of these clones could be crucial for designing specific treatment and infection control strategies (29), the objective of this work was to determine the potential underlying biological parameters. For this purpose, using a large collection of well-characterized strains with different resistance profiles from a Spanish multicenter study of bloodstream infections (19, 30) and control

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XDR epidemic high-risk clones from different hospitals, we analyzed a panel of 8 biological parameters that could potentially be involved. These parameters included the three types of bacterial motility (swimming, twitching, and swarming), the capacity to form biofilms, the production of the siderophore pyoverdine and the pyocyanin phenazine toxin, fitness (competitive growth), and spontaneous mutation rates.

MATERIALS AND METHODS

Bacterial strains, definition of resistance profiles, and molecular typing. A total of 100 well-characterized *P. aeruginosa* isolates were evaluated. Recent consensus recommendations (31) were used to define MDR (nonsusceptible to ≥3 classes) and XDR (nonsusceptible to all but 1 or 2 classes) strains, considering the following 7 antimicrobial classes: cephalosporins (ceftazidime or cefepime), penicillin–β-lactamase inhibitor combinations (piperacillin-tazobactam), monobactams (aztreonam), carbapenems (imipenem or meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin, tobramycin, or amikacin), and polymyxins (colistin). Strains susceptible to all tested antipseudomonal agents were included in the multisusceptible (MultiS) category, and those nonsusceptible to at least one agent in 1 or 2 classes were included in the moderately resistant (ModR) category. We studied the 20 XDR isolates (10.5% of all 190 isolates analyzed) recovered from 7 of the 10 Spanish hospitals participating in a 2008-2009 multicenter study of *P. aeruginosa* bloodstream infections (19, 32). The same number ($n = 20$) of isolates from each of the other three susceptibility categories (MDR, ModR, and MultiS) was randomly selected from the collection, with the only criteria being maximum coverage of different hospitals. MICs were determined in a previous study by broth microdilution following Clinical and Laboratory Standards Institute (CLSI) guidelines and breakpoints (19, 32). Likewise, the involved mutation-driven and horizontally acquired resistance mechanisms of these strains were previously characterized in detail (19, 32). Additionally, the study included a panel of 20 XDR isolates belonging to epidemic high-risk clones (ST-175 [$n = 6$], ST-111 [$n = 7$], and ST-235 [$n = 7$]) recovered from different types of infections in different hospitals and presenting multiple combinations of chromosomal resistance mechanisms and/or horizontally acquired β-lactamases (VIM-1, VIM-2, VIM-13, VIM-20, GES-5, or VEB-1) (8, 14, 17, 18, 33–35). In cases where they were unknown, genotypes were documented through multilocus sequence typing (MLST) using previously described schemes, protocols, available databases, and tools (36; http://pubmlst.org/paeruginosa). The minimum spanning tree (MST) figure was constructed using Bionumerics 7.0 software (Applied Maths, St.-Martens-Latem, Belgium).

Motility assays. (i) Swimming motility. Swimming medium (10 g/liter tryptone, 5 g/liter NaCl, and 0.3% [wt/vol] midresolution agarose) plates were inoculated with isolated colonies from an overnight culture in LB agar (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl, and 1% agar) at 37°C by use of a sharp sterile toothpick, introducing it to up to half the thickness of the medium (37).

(ii) Swarming motility. Strains were spot inoculated on swarm agar (M8 minimal medium supplemented with 1 mM $MgSO_4$, 0.2% glucose, 0.5% Bacto Casamino Acids, and 0.5% agar) plates, using 2.5-μl aliquots taken from overnight LB broth cultures (38).

(iii) Twitching motility. Isolated colonies were inoculated with a sharp sterile toothpick inserted to the bottom of twitching medium (LB agar) plates (37).

In all cases, 90-mm plates were filled with 30 ml of medium, briefly dried for 2 h, and inoculated in triplicate experiments. The plates were then wrapped with Parafilm M to prevent dehydration and incubated at 37°C for 16 h. After incubation, the zone of motility was measured. In the case of twitching medium, the agar-petri dish interface was measured. If the area to be measured was irregular, two perpendicular diameters were measured and the result was expressed as the mean of the two values.

Biofilm formation. Biofilm assays were performed following previously described procedures (39). Briefly, 10^5 cells in stationary phase were inoculated into each of four wells per strain in a microtiter plate containing fresh LB medium, to a final volume of 100 μl per well. After incubation for 24 h at 37°C, microtiter plates were gently rinsed with tap water. After removing all planktonic cells, plates were air dried and stained with 125 μl of a 0.1% crystal violet solution per well for 10 min. The plates were gently rinsed with water and air dried again. The dye was solubilized with 200 μl of 30% acetic acid for 25 min at room temperature. Once mixed by pipetting, 125 μl of each well's contents was individually transferred to a clear flat-bottom 96-well plate. Finally, absorbance was measured at 590 nm. The final expressed values are means for four replicates.

Determination of spontaneous mutant frequencies. The frequencies of mutation to rifampin (300 μg/ml) resistance were determined according to previously described protocols (40). For each strain, independent aliquots containing approximately 10^3 cells were inoculated into five flasks containing 1 ml of Mueller-Hinton broth (each) and incubated at 37°C and 180 rpm for 16 to 18 h. Serial 1:10 dilutions were then plated on Mueller-Hinton agar plates and Mueller-Hinton agar plates supplemented with 300 μg/ml of rifampin. Mutant frequencies were calculated by dividing the median numbers of mutants by the median numbers of total cells in the five independent cultures. The breakpoints used to define strong and weak mutator strains were rifampin resistance mutant frequencies of $\geq 2 \times 10^{-7}$ (20-fold higher than that of wild-type PAO1) and $\geq 5 \times 10^{-8}$ (5-fold higher than that of PAO1), respectively (41). Wild-type PAO1 and its *mutS*-deficient mutator derivative (PAOMS) were used as controls (40).

Pigment production. Production of pyocyanin and pyoverdine was quantified as described previously (28). Briefly, bacterial strains were grown at 37°C in *Pseudomonas* ACC broth for 40 h. At this time, bacteria were pelleted by centrifugation, and the amount of the blue pigment pyocyanin was evaluated by measuring the absorbance of the supernatants at 690 nm. The amount of pyoverdine was measured by fluorescence emission, by exciting the supernatants at 400 nm and measuring the emission at 460 nm. Each experiment was performed in triplicate.

Determination of *in vitro* fitness through flow cytometry-coupled competitive growth experiments. A new *in vitro* competition assay was implemented from previously described protocols (27) by adding flow cytometry as the cell-differentiating method. This novel protocol does not require time-consuming CFU enumeration and the presence of selectable antibiotic resistance markers to differentiate the tested strains. For this purpose, wild-type PAO1 was fluorescently tagged at the *att* intergenic neutral chromosomal locus with *gfp* (encoding green fluorescent protein [GFP]), carried in mini-Tn*7* constructs also containing a gentamicin resistance gene as a resistance marker, as described previously (42). Exponentially growing cells of the corresponding clinical strain and GFP-tagged wild-type PAO1 (GFP-PAO1) in LB broth were then mixed in a 1:1 ratio and diluted in 0.9% saline solution. Approximately 10^3 cells from each of the mixtures were inoculated into three 10-ml LB broth flasks and grown at 37°C and 180 rpm for 16 to 18 h, corresponding to approximately 20 generations. After incubation, each mixed culture was diluted in saline solution to an optical density at 600 nm (OD_{600}) of 0.1. Fluorescence-assisted flow cytometry was used to distinguish between the wild-type PAO1 population producing GFP and the clinical strain populations. The experiments were carried out using a Beckman-Coulter Epics XL instrument. GFP-PAO1 was excited using a 488-nm blue laser and detected using a 525/40-nm filter. Data were processed with the provided EXPO 32 software. The competition index (CI) was defined as the final ratio between each evaluated strain and GFP-PAO1. In the early stages, this method was validated by comparing results with those of CFU enumeration as the gold standard method (27). For this purpose, competition experiments were performed between 20 gentamicin-susceptible clinical strains and GFP-PAO1 (gentamicin resistant). CIs were determined by plating strains on LB agar and LB agar supplemented with 15 μg/ml gentamicin, in

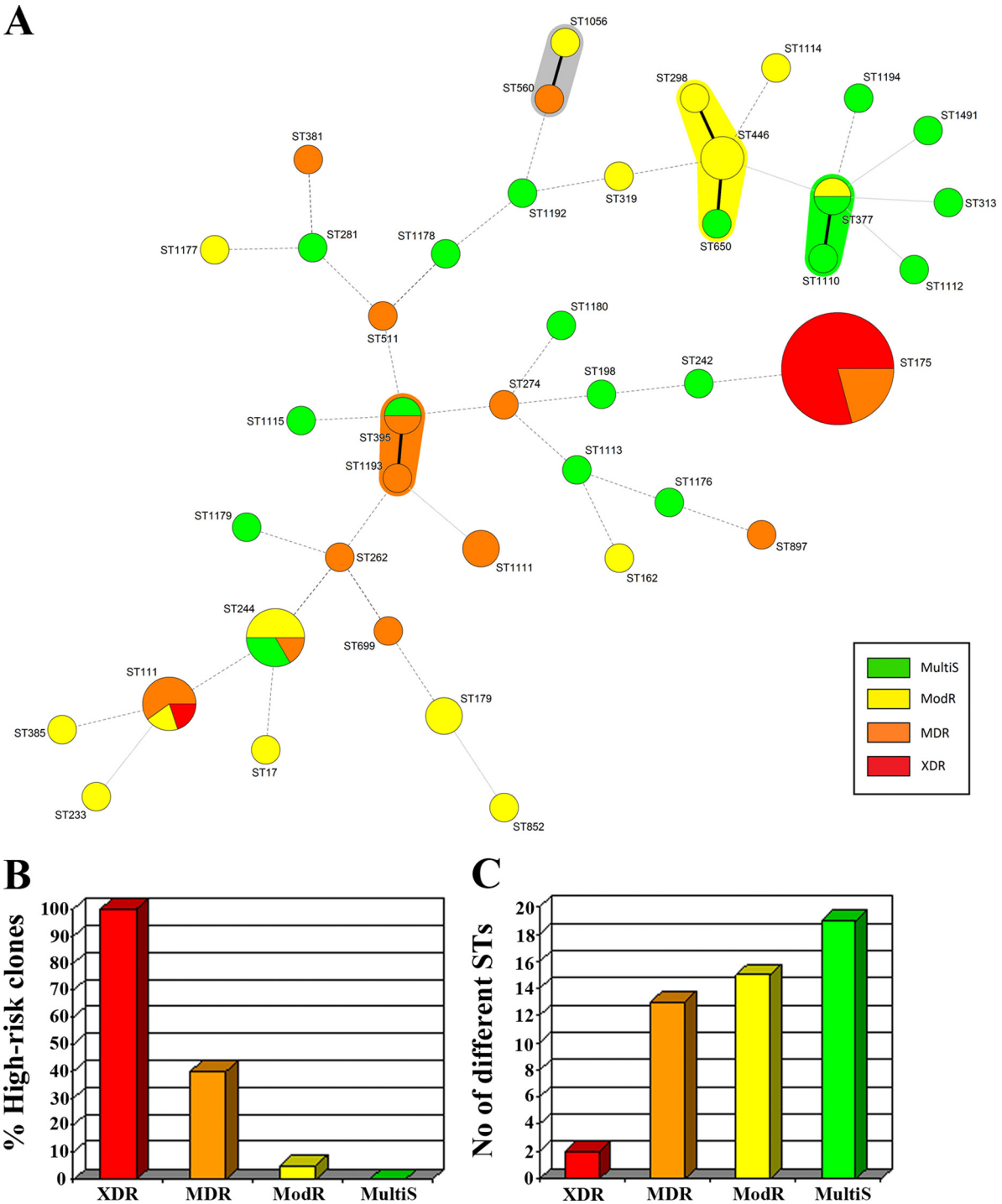


FIG 1 (A) Minimum spanning tree of the 80 *P. aeruginosa* bloodstream isolates from the Spanish multicenter study. Each of the 4 resistance profiles (XDR, MDR, ModR, and MultiS) is represented by a different color. Wide lines represent single-locus variants, and dotted lines represent multilocus variants. The sizes of the circles represent the numbers of isolates found with the respective STs. (B) Percentages of isolates belonging to high-risk clones (ST-111, ST-175, or ST-235) among XDR, MDR, ModR, and MultiS isolates. (C) Clonal diversity among XDR, MDR, ModR, and MultiS isolates.

parallel with the flow cytometry assays, validating the concordance of both procedures. Three independent experiments were performed for each clinical isolate, and mean CI values were recorded.

Data analysis. GraphPad Prism 5 software was used for graphical representation and statistical analysis. Quantitative variables were compared using the Mann-Whitney U test or the Student *t* test, as appropriate. Categorical variables were compared using the χ^2 test. A *P* value of <0.05 was considered statistically significant. For hierarchical clustering of the strains according to the 8 measured variables, the obtained values for each isolate were divided by the median values for all isolates and then analyzed with software available at the Eisen lab website (43; http://rana.lbl.gov/EisenSoftware.htm [accessed 10 January 2013]).

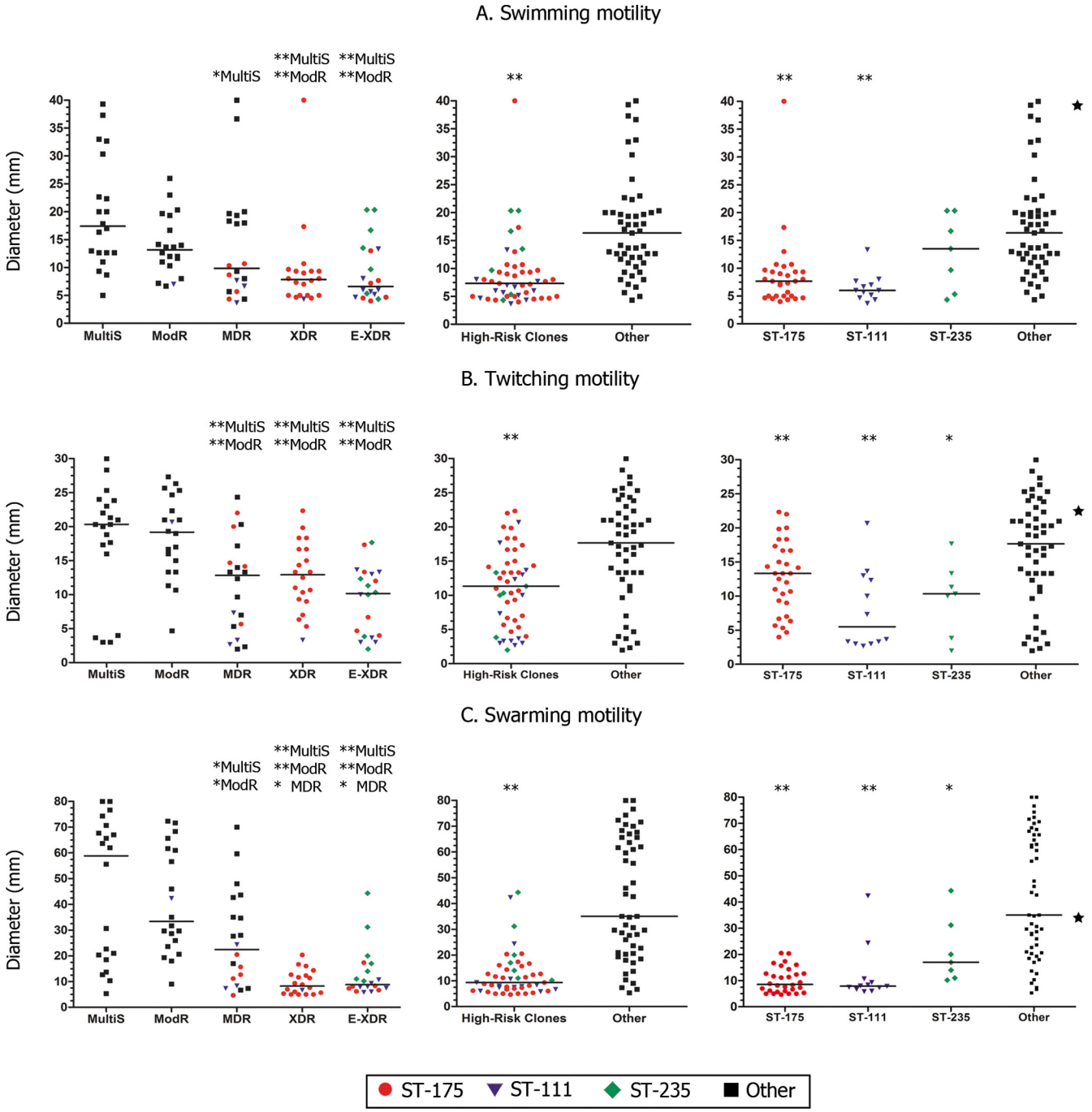


FIG 2 Results of swimming (A), twitching (B), and swarming (C) motility assays. (Left) Comparative analyses of the five 20-isolate groups (MultiS, ModR, MDR, XDR, and E-XDR) studied. (Center) Comparative analyses of high-risk clones (all together) versus other clones. (Right) Comparative analyses of each of the high-risk clones (ST-175, ST-111, and ST-235) versus other clones. *, $P < 0.05$; **, $P < 0.01$. The values for the reference strain PAO1 are indicated with black stars.

RESULTS AND DISCUSSION

Resistance profiles and *P. aeruginosa* epidemic high-risk clones. We analyzed a panel of 20 isolates from each of four established resistance categories, i.e., XDR, MDR, ModR (resistant to 1 or 2 antimicrobial classes), and MultiS (susceptible to all antipseudomonal agents), recovered from a multicenter study of *P. aeruginosa* bloodstream infections performed in 10 Spanish hospitals. The correlations between the four resistance categories and the epidemic high-risk clones are shown in Fig. 1. All 20 XDR isolates belonged to high-risk clones (ST-175 or ST-111), whereas clonal diversity was much higher for the 20 MDR isolates, which included 8 isolates belonging to high-risk clones and 12 isolates belonging to 11 different sporadic clones. Moreover, high-risk clones were documented for only one of the ModR isolates and none of the MultiS isolates, among which clonal diversity was highest (Fig. 1). Overall, 29 of the 80 isolates studied belonged to

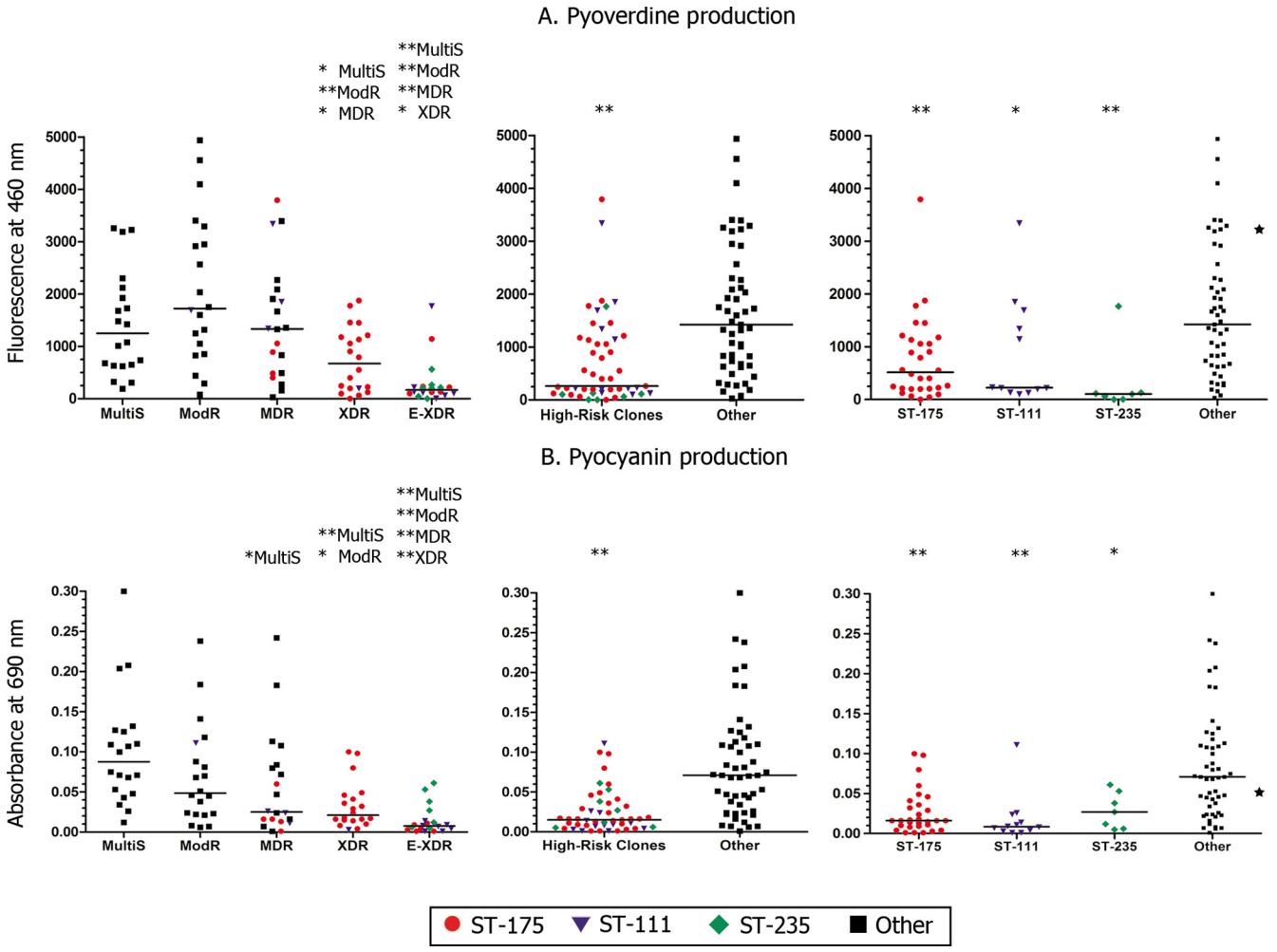


FIG 3 Results of pyoverdine (A) and pyocyanin (B) production assays. (Left) Comparative analyses of the five 20-isolate groups (MultiS, ModR, MDR, XDR, and E-XDR) studied. (Center) Comparative analyses of high-risk clones (all together) versus other clones. (Right) Comparative analyses of each of the high-risk clones (ST-175, ST-111, and ST-235) versus other clones. *, $P < 0.05$; **, $P < 0.01$. The values for the reference strain PAO1 are indicated with black stars.

high-risk clones, including 24 ST-175 and 5 ST-111 isolates, and these were detected in 8 of the 10 hospitals, with a wide geographical distribution covering all 4 regions participating in the study. Isolates belonging to the third international high-risk clone, ST-235, were not detected in our multicenter study of bloodstream infections, but other recent studies also detected this clone in Spain, linked either to a large outbreak of GES-5 class A carbapenemase-producing *P. aeruginosa* in a hospital in Madrid (17) or to the class B carbapenemase VIM-13, which is autochthonous to the Balearic Islands (33). To widen the diversity and spectrum of high-risk clone isolates included in the subsequent analysis of biological markers, we added another, fifth panel (designated E-XDR), composed of 20 previously characterized XDR isolates belonging to the three major epidemic high-risk clones (ST-175 [$n = 6$], ST-111 [$n = 7$], and ST-235 [$n = 7$]), producing outbreaks in several different hospitals, and presenting multiple different combinations of chromosomal resistance mechanisms and/or horizontally acquired β-lactamases.

Defective motility in widespread epidemic high-risk clones. It is widely accepted that the diverse repertoire of bacterial motility systems, including swimming (flagellum-mediated motility in liquid media), twitching (type IV fimbria-mediated motility with surface adherence), and swarming (flagellum-mediated, coordinated movement across surfaces) motility, plays a pivotal role in the colonization of fluids and surfaces, including those found in the nosocomial environment or the patient's epithelial tissues (44). Motility systems are also well-established virulence determinants, playing a major role in tissue invasion during infections (45). Therefore, differentially expressed motility systems were strong candidates to be involved in the clonal success of *P. aeruginosa* high-risk clones. Figure 2 shows the results for swimming, twitching, and swarming motility assays. As shown, no statistically significant differences were observed for any of the three motility systems between clonally diverse MultiS and ModR isolates, indicating that single resistance mechanisms of *P. aeruginosa* clinical strains do not have a uniform impact on swimming, twitching, or swarming motility. On the other hand, in contrast to our initial expectations, MDR/XDR high-risk clones were found to be associated significantly with defective swimming, twitching, and swarming motility. Moreover, this association was significant for all three high-risk clones and all three motility systems individually, except for the case of ST-235 and swimming motility (Fig. 2).

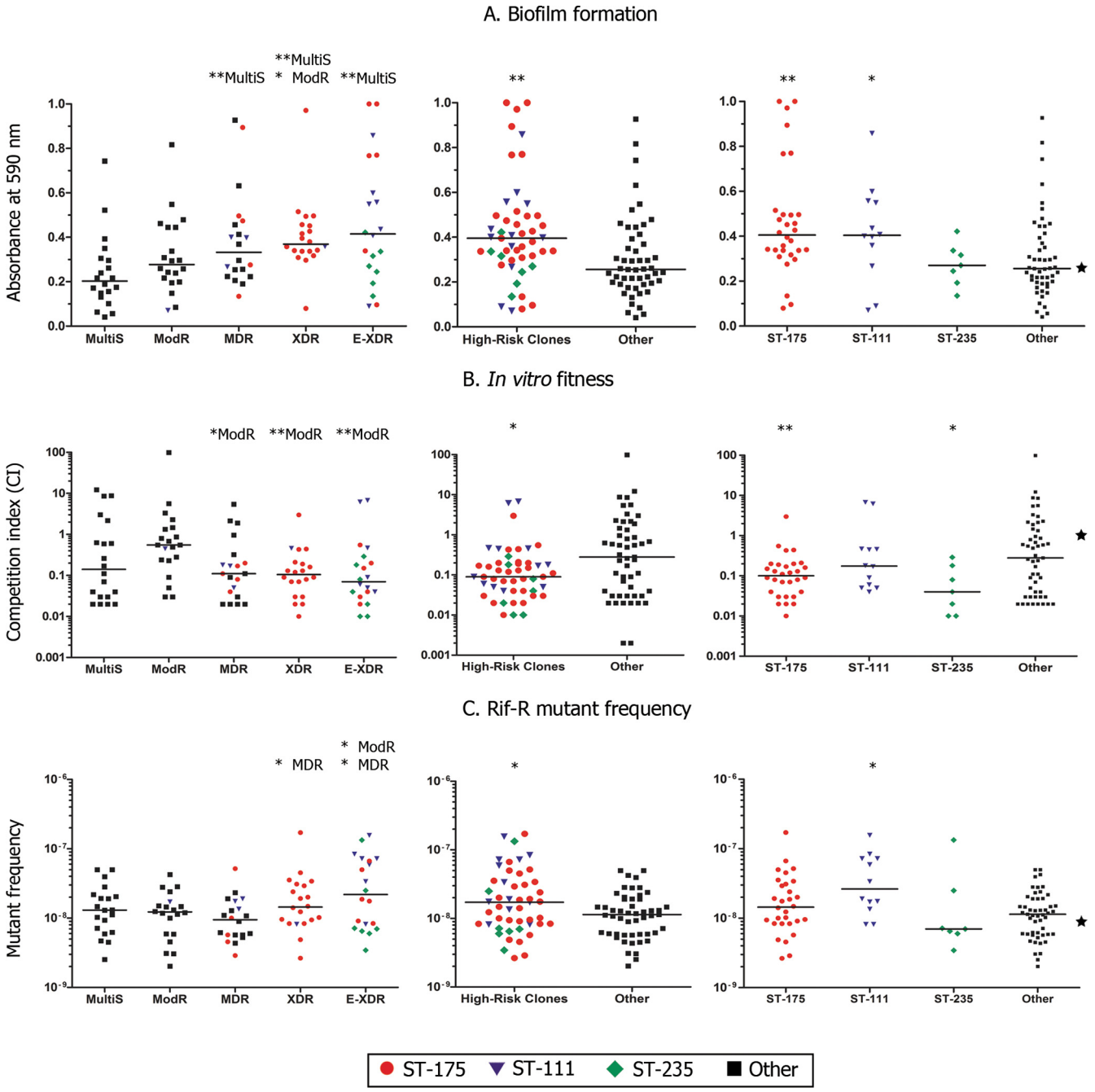
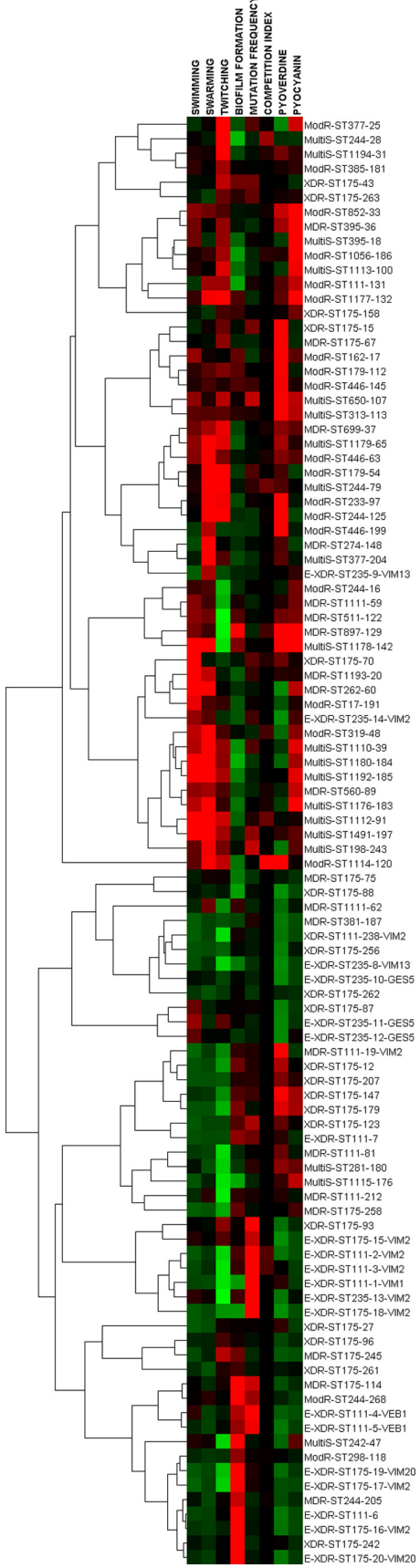


FIG 4 Results of biofilm formation (A) and *in vitro* fitness (B) assays and rifampin resistance (Rif^r) mutant frequencies (C). (Left) Comparative analyses of the five 20-isolate groups (MultiS, ModR, MDR, XDR, and E-XDR) studied. (Center) Comparative analyses of high-risk clones (all together) versus other clones. (Right) Comparative analyses of each of the high-risk clones (ST-175, ST-111, and ST-235) versus other clones. *, $P < 0.05$; **, $P < 0.01$. The values for the reference strain PAO1 are indicated with black stars.

Whether this strong association of high-risk clones with defective motility is driven by the activation of specific regulatory pathways in these lineages, negative selection of metabolically costly motility systems, or positive selection of mechanisms to evade the host immune system (i.e., fimbriae and flagella are key targets for the recognition of pathogens by the immune system) still needs to be explored. In any case, the presented results clearly indicate that none of the motility systems are required for the successful spread of these global clones.

Defective pigment production in widespread epidemic high-risk clones. One of the most characteristic features of *P. aeruginosa* is the production of pigments. Indeed, in addition to conferring a distinctive blue-green color to *P. aeruginosa* colonies, pyocyanin (a redox-active phenazine toxin) and pyoverdine (a siderophore) are well known to play a major role in bacterial physiology and pathogenesis (46, 47). Therefore, differential production of pigments was also a strong candidate to be involved in the clonal success of *P. aeruginosa* high-risk clones. Figure 3 shows the



results of pyocyanin and pyoverdine production assays. Once more, significant differences were not observed for either pyocyanin or pyoverdine between MultiS and ModR isolates. On the other hand, again in contrast to our initial expectations, MDR/XDR high-risk clones were found to be associated significantly with defective pigment production (Fig. 3). Moreover, this association was significant for both pigments in all three high-risk clones individually, indicating that defective pyocyanin and pyoverdine production is a common signature of these globally spread strains.

Increased biofilm formation in widespread epidemic high-risk clones. One of the major bacterial strategies for colonizing nearly all types of environments, from natural ecosystems to nosocomial settings and human hosts, is the development of structured multicellular communities embedded in a polymeric matrix, known as biofilms (48). Bacterial biofilms are highly resistant to antibiotics and effectors of the immune system (such as antibodies and phagocytes) and represent a major source of persistent infections (49). Therefore, an enhanced capacity to form biofilms was also a strong candidate to be involved in the clonal success of *P. aeruginosa* high-risk clones. Figure 4A shows the results of biofilm production assays. Once again, biofilm formation was not significantly different in MultiS and ModR isolates. However, in contrast to motility and pigment production, biofilm formation was significantly enhanced globally in high-risk clones, and individually for ST-175 and ST-111 isolates (Fig. 4A). Therefore, the results so far indicate that MDR/XDR epidemic high-risk clones are associated with defective motility and pigment production but increased biofilm formation, which, interestingly, are attributes that resemble the phenotypic signature observed during *P. aeruginosa* adaptation in chronic infections (49, 50). Thus, the adaptive processes for long-term persistence in chronic infection and global clonal success in the nosocomial environment appear to have common elements, perhaps through one of the several potential regulatory networks connecting all these elements, including antibiotic resistance, motility, pigment production, and biofilm formation (23).

***In vitro* fitness is not higher in widespread epidemic high-risk clones.** Certainly, a major issue to consider in analyzing clonal success is bacterial fitness. It is well known that antibiotic resistance mechanisms may impose a fitness cost but also that this cost may be minimized through compensatory mutations (21, 26). Although fitness is a relative parameter that obviously will be highly dependent on the specific environmental conditions faced by microorganisms, standardized *in vitro* competitive growth assays are useful for estimating the metabolic cost of antibiotic resistance mechanisms (21). Figure 4B shows the results for competition assays. Once again, significant differences were not observed between clonally diverse MultiS and ModR isolates, indicating that single resistance mechanisms of *P. aeruginosa* clinical strains do not have a uniform impact on fitness. However, competition indexes were significantly lower for high-risk clones, showing that

FIG 5 Hierarchical clustering of the strains according to the 8 measured variables (swimming, twitching, and swarming motility, biofilm formation, production of pyoverdine and pyocyanin, spontaneous [rifampin resistance] mutant frequencies, and *in vitro* CI), using the software available at the Eisen lab website (http://rana.lbl.gov/EisenSoftware.htm). Green and red indicate that the values are lower and higher than the median, respectively. The acquired β-lactamases produced are also indicated.

enhanced *in vitro* fitness does not explain the clonal success of these widely spread clones.

Increased spontaneous mutation rates in some widespread epidemic high-risk clones. A major adaptive strategy of bacterial pathogens facing new environments or adverse conditions is the selection of variants with increased mutation rates, known as mutators. Indeed, variants with highly increased mutation rates (strong mutators), due to a defective DNA mismatch repair system, are found to be very frequent in *P. aeruginosa* chronic infections linked to antibiotic resistance, although their prevalence in acute nosocomial infections is very low (40, 41, 48). Moderately increased mutation rates (weak mutator phenotypes) were also shown to be selected positively in chronic infections linked to antibiotic resistance (51). However, so far, there was no available information on the mutation rates of *P. aeruginosa* high-risk clones. Interestingly, as shown in Fig. 4C, spontaneous mutant frequencies were found to be significantly higher for high-risk clones, although individually, differences were significant only for ST-111 isolates. Moreover, while none of the 100 isolates were classified as strong mutators, a significantly larger proportion of weak mutators was detected among high-risk clones than among the other clones (20.4% versus 3.9%; $P = 0.01$). Therefore, weak mutator phenotypes could play a role in the high adaptability required for global dissemination of high-risk clones.

Concluding remarks. In summary, our results indicate that *P. aeruginosa* high-risk clones are significantly associated with a defined set of biological parameters which include increased biofilm formation and mutant frequencies but reduced motility (twitching, swimming, and swarming), pigment (pyoverdine and pyocyanin) production, and *in vitro* fitness (Fig. 5). The defined biological markers of *P. aeruginosa* high-risk clones, which present certain similarities with those resulting from adaptation to chronic infections, could be useful for the future design of specific treatment and infection control strategies. Moreover, while the investigated biological parameters were chosen for their potential relevance to clonal success, the presence of specific virulence traits in high-risk clones that determine a higher or lower disease severity still needs to be explored in further studies.

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