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Antimicrobial Tolerance in Biofilms

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Abstract

The tolerance of microorganisms in biofilms to antimicrobial agents is examined through a meta-analysis of literature data. A numerical tolerance factor comparing the rates of killing in the planktonic and biofilm states is defined to provide a quantitative basis for the analysis. Tolerance factors for biocides and antibiotics range over three orders of magnitude. This variation is not explained by taking into account the molecular weight of the agent, the chemistry of the agent, the substratum material, or the speciation of the microorganisms. Tolerance factors do depend on the areal cell density of the biofilm at the time of treatment and on the age of the biofilm as grown in a particular experimental system. This suggests that there is something that happens during biofilm maturation, either physical or physiological, that is essential for full biofilm tolerance. Experimental measurements of antimicrobial penetration times in biofilms range over orders of magnitude, with slower penetration (>12 min) observed for reactive oxidants and cationic molecules. These agents are retarded through the interaction of reaction, sorption, and diffusion. The specific physiological status of microbial cells in a biofilm contributes to antimicrobial tolerance. A conceptual framework for categorizing physiological cell states is discussed in the context of antimicrobial susceptibility. It is likely that biofilms harbor cells in multiple states simultaneously (e.g., growing, stress-adapted, dormant, inactive) and that this physiological heterogeneity is an important factor in the tolerance of the biofilm state.

EXAMPLES OF REDUCED BIOFILM SUSCEPTIBILITY

Tolerance to antimicrobial agents is a common feature of microbial biofilm formation (1–7). Table 1 presents a few examples of biofilm tolerance to biocides and antiseptics, and Table 2 summarizes some examples of antibiotic tolerance in biofilms. Neither of these listings is comprehensive, but these two data sets can be analyzed to gain insight into the factors that influence biofilm tolerance. The examples have been selected to illustrate the wide variety of microbial species, growth environments, and antimicrobial chemistries for which biofilm reduced susceptibility has been reported. The short list in Table 1 encompasses studies designed to mimic biofilms in dental plaque, hot tubs, paper mills, drinking water, household drains, urinary catheters, food processing plants, cooling water systems, and hospitals. These examples employ a range of individual and mixed species biofilms and diverse biocidal chemistries including halogens, phenolics, quaternary ammonium compounds, aldehydes, a plant essential oil, and peroxides. The studies captured in Table 2

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cover 19 antibiotics and 9 organisms that include aerobic bacteria, strict anaerobes, and a fungus.

Biofilm reduced susceptibility is quantified in Tables 1 and 2 by a tolerance factor, *TF*, defined as:

$$TF = (LR_P * t_B * C_B / LR_B * t_P * C_P)$$

where C_P and C_B denote planktonic and biofilm dose concentration, respectively, t_P and t_B denote planktonic and biofilm dose duration, respectively, and LR_P and LR_B denote the measured log reduction in planktonic and biofilm populations, respectively.

TF compares the rate of killing in the planktonic and biofilm states. For example, a value of *TF* = 10 means that biofilm killing is 10 times slower than in the planktonic condition. A quick inspection of Tables 1 and 2 reveals that the tolerance factor ranges widely, from a value of 1.0 (no difference at all between suspended and sessile susceptibility) to a value of more than 1,000.

FACTORS INFLUENCING BIOFILM SUSCEPTIBILITY

One of the challenges of understanding biofilm tolerance is the large number of factors that likely influence the susceptibility in a particular biofilm. Some of the factors that could be important are antimicrobial chemistry, substratum material, areal cell density or thickness, biofilm age, microbial speciation, and medium composition. Here I attempt to shed some light on some of these factors by meta-analyses of the literature.

Antimicrobial Chemistry

When the tolerance factors for biocides reported in Table 1 are regressed against the molecular weight of the antimicrobial agent, no correlation whatsoever is apparent (Fig. 1A; $R^2 = 0.0007$). Indeed, this value of R^2 suggests that none of the considerable variation in *TF* can be attributed to the size of the antimicrobial molecule itself. A similar analysis of the tolerance factors for antibiotics (Table 2) also reveals no correlation (Fig. 1B; $R^2 = 0.012$). There are no reports in the literature demonstrating that antimicrobial size is a predictor of efficacy against biofilms.

TF ranges widely even for a single antimicrobial agent. For example, values of *TF* for tobramycin measured against just one bacterium, *Pseudomonas aeruginosa*, run from 1.5 to 265. *TF* values for ciprofloxacin, measured against four different bacteria, range from 3.5 to 2,048. It will be seen shortly that the rate of biofilm killing by chlorine ranges over three orders of magnitude even when scaled for the dose concentration. These observations suggest that the numerical value of *TF* is not specific to a particular antimicrobial agent. Put another way, at least at this point in time, the chemistry of a particular antimicrobial does not allow us to predict its relative efficacy against a biofilm.

At the risk of redundancy, it is important not to extrapolate a *TF* value pulled from the tables compiled here to some other system. It is to be expected that if more measurements were available, we would find that *TF* values for every antimicrobial range widely.

Substratum Material

The data compiled in Tables 1 and 2 reflect measurements made using biofilms formed on a wide variety of materials: polystyrene, glass, stainless steel, cellulose acetate or nitrate, polycarbonate, silicone, polyvinyl chloride, rubber, polyester, and hydroxyapatite. Though analysis of variance of these data (plotted in Fig. 2) indicates a borderline statistically significant difference between the five groups ($p = 0.053$), I suspect that the root of this difference is in methodology rather than material. The polystyrene group, which has somewhat higher *TF* values, is all data from multiwell plates. Most of the data collected in plate assays derive from a series of antimicrobial concentrations as opposed to kill data in time. This method can produce very high *TF* values with antibiotics when delivered at extremely high (and not physiologically relevant) concentrations. Inspection of the data shows that *TF* ranges by two orders of magnitude for a given material (Fig. 2). For example, tolerance factors reported for biofilms grown on stainless steel ($n = 8$) range from 1.1 to 767.

There may be occasional situations in which the substratum material does influence biofilm accumulation and antimicrobial tolerance. For example, whereas iodine was relatively effective at killing *Listeria* on stainless steel (*TF* = 1.7), it was ineffective against the same strain when biofilms were formed on Buna rubber (*TF* = 70) (8). Buna rubber was shown to have an independent bacteriostatic effect. Biofilms formed on mild steel, in which some corrosion of the metal was evident, were less susceptible to killing by monochloramine than biofilms on stainless steel (9). These examples suggest that the substratum material is most likely to influence biofilm susceptibility when it leaches or corrodes.

Cell Density

When the tolerance factors for biocides and antiseptics tabulated in Table 1 are regressed against the untreated control biofilm areal cell density (measured in units of $\log_{10}$ CFU cm^{-2}), a clear correlation emerges (Fig. 3; $R^2 = 0.629$). To put these values in terms of the approximate thickness of the biofilm, a biofilm of 6.0 $\log_{10}$ CFU cm^{-2} corresponds roughly to a sparse monolayer, whereas the most massive biofilm in this data set (9.9 $\log_{10}$ CFU cm^{-2}) was nearly 1 mm thick. This result shows that tolerance to biocides depends on the extent of biofilm accumulation.

There are few biocides for which there is sufficient data available to perform an agent-specific analysis of the role of biofilm cell density in susceptibility. Chlorine is one such agent, and Figure 4 presents a correlation ($R^2 = 0.757$) that reinforces an important role for the extent of biofilm accumulation prior to treatment in determining the efficacy of a chlorine dose. This analysis includes data from nine independent investigations using *Staphylococcus*, *Pseudomonas*, *Listeria*, *Salmonella*, and mixed-species biofilms. There is also an important dependence of biofilm antibiotic tolerance on the cell density of the biofilm. This is most clearly demonstrated in investigations which have challenged biofilms at different stages of development with the same antibiotic dose. Older, thicker biofilms are

invariably less susceptible than younger, less dense biofilms (Fig. 5). The overall correlation of log reduction with cell density is poor in this case (R^2 = 0.125), but the effect within an investigation is obvious and consistent.

Age

Several investigations have compared the efficacy of identical antimicrobial challenges against biofilms of different ages. Within a given experimental system, bio-films tend to become less susceptible as they age (Fig. 6), though here again the overall correlation is not strong (R^2 = 0.217). Assuming a first order process, the characteristic time (expressed as a half-life) for biofilm tolerance to develop as determined from these data sets was 2.7 ± 2.0 days (n = 12). This suggests that, at least *in vitro*, biofilm tolerance manifests over a timescale of a few days. This result also provides an important clue that the biological state of the organisms in a biofilm is a key factor in determining their susceptibility.

Biofilm age and biofilm cell density are usually strongly correlated. The effects of these two parameters are therefore easily confounded. Is the difference in susceptibility between 2-day-old and 7-day-old biofilms (10) a function of age or a function of the substantial difference in biofilm accumulation at these two time points? Here I analyze one data set where, fortuitously, it is possible to separate these two parameters. Wolcott et al. (11) reported on the challenge of *Staphylococcus aureus* biofilm with gentamicin. During more than 100 h of the maturation of the biofilm there was little change in the biofilm cell density (Fig. 7A). Biofilms removed at different ages were treated with gentamicin, and the log reduction in viable counts was determined. This log reduction did not correlate with the untreated control biofilm cell density (R^2 = 0.087). There was correlation between the biofilm susceptibility to gentamicin and biofilm age (Fig. 7B; R^2 = 0.470).

Though the preceding example indicates a more important role for biofilm age than for cell density, in general it is very difficult to uncouple the individual contributions of age and density to biofilm tolerance.

Species Composition

In this section I explore the role of the microbial composition of a biofilm on its antimicrobial tolerance. Tolerance factors, for both biocides and antibiotics, are grouped by phylum in Figure 8. There is no statistically significant difference between the mean values of *TF* for any of the phyla (p = 0.26 by analysis of variance). For the three phyla for which there are four or more data points (*Firmicutes*, *Proteobacteria*, *Actinobacteria*), *TF* ranges over at least two orders of magnitude. One thing these data suggest is that tolerance is not specific to any particular subgroup of microorganisms. Indeed, reduced biofilm susceptibility appears to be a broadly distributed capability across the microbial world.

Medium Composition

Antimicrobial susceptibility can be very sensitive to the composition of the medium used in the assay. I was not able to devise an informative way to test for effects of medium composition on *TF* values. To underscore the dramatic influence medium composition can play, Figure 9 presents some measurements made with young *Escherichia coli* or *P.*

aeruginosa biofilms. At this early stage of development, antibiotics can be very effective against the bacteria under certain culture conditions. However, changes in the medium can drastically alter bacterial susceptibility. For example, 6-h-old *E. coli* biofilms are decimated by kanamycin when challenged on LB medium (8.4 log reduction) but scarcely affected when the medium is supplemented with glucose (1.2 log reduction). A similarly dramatic effect is seen for ampicillin treatment, except that it is exactly the reverse: on LB medium ampicillin is ineffective (1.4 log reduction), whereas the addition of glucose greatly enhances killing (7.6 log reduction). Analogous alterations in antibiotic susceptibility can be seen in 4-h-old *P. aeruginosa* biofilms exposed to tetracycline or tobramycin on different media (Fig. 9). For each agent there are conditions under which they are very effective and conditions under which they are ineffective. These conditions are not the same for the different antibiotics. These data lead to the hypothesis that medium composition influences microbial physiology, which in turn alters antimicrobial susceptibility.

Summary

What has been shown so far is that there is no discernable generalized role of antimicrobial size, antimicrobial chemistry, substratum material, or microbial species composition on the quantitative level of tolerance established during biofilm formation. Only areal cell density and biofilm age partially correlate with antimicrobial tolerance. This suggests that there is something that happens during biofilm maturation, either physical or physiological, that is essential for full biofilm tolerance. Case study results also point to an important role for medium composition, and hence physiology, in biofilm tolerance. Another way to say this is that the details of how the biofilm is grown for a particular test are likely to be more important than the choice of antimicrobial agent or microorganism.

MECHANISMS OF BIOFILM ANTIMICROBIAL TOLERANCE

Antimicrobial Depletion

One simple and possibly underappreciated mechanism of biofilm protection is depletion of the antimicrobial agent in the fluid bathing the biofilm. The antimicrobial could be depleted either by reaction in the fluid phase, by reaction with the biofilm or attachment substratum, or by sorption to constituents of the biofilm or substratum material. This mechanism is especially plausible in systems with a relatively high surface area to volume ratio, such as a 96-well microtiter plate. In this type of system, the demand exhibited by the biofilm could quickly reduce the dissolved concentration of antimicrobial.

The obvious way to control for antimicrobial depletion is to assay the bulk fluid during the course of treatment, or at least before and after the exposure period, to test whether the antimicrobial concentration is sustained. This is not typically done.

Since the surface area to volume ratio is a critical physical characteristic of a system, determining the potential for antimicrobial depletion, and since most of the data sets in Tables 1 and 2 include details permitting calculation of this ratio, a quantitative analysis can be conducted. When the tolerance factors in Table 1 are regressed against the surface area to volume ratio, no correlation is apparent ($R^2 = 0.022$). Neither do the biofilm *TF*s for antibiotics in Table 2 correlate with the surface area to volume ratio of the biofilm test

system (R^2 = 0.010). What these analyses indicate is that antimicrobial depletion is probably not a general cause of biofilm tolerance in *in vitro* models.

Penetration

The extent of antimicrobial penetration into a biofilm is expected to depend on biofilm thickness, effective diffusivity of the agent in the biofilm, reactivity of the agent in the biofilm, the sorptive capacity of the biofilm for the agent, the dose concentration and dose duration, and external mass transfer properties (12). In other words, this is a complex interaction and problem. A good starting place is to examine actual measurements of antimicrobial penetration in biofilms.

A survey of experimentally measured penetration times of antimicrobial agents in biofilms is presented in Figure 10. This data set excludes measurements made using diffusion chambers in which the biofilm is sandwiched between two compartments. These approaches can be useful for determining whether penetration occurs but are not appropriate for determining absolute penetration times, because the time constants are dependent on the device geometry. The measurements reported in Figure 10 were made using microelectrodes, time lapse microscopy of fluorescent-tagged drugs, total internal reflection spectroscopy, and time lapse microscopy of fluorescence loss from cells preloaded with a fluorophore.

The penetration times in Figure 10 range from a fraction of a minute to almost a full day. It is tempting to judge some of these as fast and others as slow, but keep in mind that the important comparison to be made is between the dose duration and the penetration time. A penetration time of 30 min could be fast if the dose duration is 8 h and slow if the dose duration is 3 min.

Penetration times do not increase with the molecular weight of the antimicrobial as intuition might suggest. Indeed, one thing that can be inferred from Figure 10 is that even large antibiotics or antimicrobial peptides can penetrate a biofilm within a few minutes. Some examples of large agents that access the interior of a biofilm relatively quickly include vancomycin (0.5 min), daptomycin (1.5 min), and nisin (4 to 10 min).

There are two groups of agents, circled in Figure 10, with measured penetration times longer than 12 min. The antimicrobials in the first group (lower molecular weight) are all reactive oxidants: chlorine, chlorine dioxide, monochloramine, and hydrogen peroxide. The agents in the second group (higher molecular weight) are mostly cationic molecules including quaternary ammonium compounds, such as cetylpyridinium chloride and benzalkonium chloride, and an aminoglycoside antibiotic. The retarded penetration of these agents into the biofilm derives from the reaction or sorption of the agent in the biofilm as it diffuses. Halogens react with unchar-acterized components of biomass and are neutralized. Hydrogen peroxide is destroyed by the action of catalase. Agents with a positive charge likely bind to negatively charged polymers or to cell surfaces, delaying penetration. Retarded penetration due to reaction and sorption has been analyzed by mathematical models (12, 13).

When considering agents that are subject to reaction or sorption in the biofilm, it is anticipated that the rate of penetration will depend on the applied concentration. This prediction is borne out by the subset of data plotted in Figure 11. This analysis shows that agents such as chlorine, peracetic acid, and tobramycin (all members of the circled groups in Fig. 10) penetrate a given biofilm faster as the applied concentration is increased. The slope of the regressed line in Figure 11 is close to −1. This tells us that penetration time for these agents is inversely proportional to dose concentration. For example, a dose concentration that is 10 times higher will result in a penetration time that is one 10th as long. This is not expected to be true of antimicrobials that do not react or sorb in the biofilm. The 50% penetration time for a noninteracting solute is predicted to be independent of the applied concentration.

The preceding analysis and discussion is helpful for gaining insight into the fundamental phenomenon of antimicrobial penetration in a biofilm, but it does not tell us if retarded penetration actually limits antimicrobial efficacy in practice. The most likely situation for incomplete penetration to occur is when reactive oxidants are delivered at relatively low concentrations to thick bio-films for brief dose durations. Antibiotics likely penetrate biofilms *in vivo* because dose durations are relatively long. Another argument for penetration of antibiotics, including the sticky aminoglycosides, is that they result in log reductions *in vivo* that indicate access to most of the bacteria. For example, a classic clinical study of inhaled tobramycin in cystic fibrosis patients reported log reductions of *P. aeruginosa* in sputum of slightly greater than 2 after 2 weeks of therapy (14). This tells us that the drug reached 99% of the bacteria. Even in applications in which the dose duration is brief, for example, a mouth rinse treating dental plaque, penetration of the antimicrobial may not be limiting. Corbin (15) found no correlation between the clinical efficacy of mouth rinse active ingredients and their *in vitro* penetration time.

Physiology

Microorganisms in biofilms may be tolerant to antimicrobial agents because they enter less susceptible physiological states. For example, it is widely appreciated that microorganisms in the stationary phase of a batch planktonic culture, which may be slow-growing or non-growing and may be less metabolically active than growing cells, can be less sensitive to killing by antimicrobials. A few research studies have compared killing in exponential phase, stationary phase, and biofilms (Fig. 12). Though this analysis lacks sufficient data to make a strong conclusion, it suggests that whereas exponential phase planktonic cells are clearly less susceptible than biofilms cells, stationary phase planktonic cells are not consistently different from biofilm cells. The terminology of batch planktonic cultures is probably inadequate as a basis for characterizing the physiological heterogeneity within a biofilm.

A wide variety of terms have been used to characterize the physiological state of a microbial cell: exponential phase, stationary phase, lag phase, nongrowing, stressed, adapted, inactive, viable but nonculturable, persister, dormant. Figure 13 presents a simplified categorization of physiological states based on discrimination of three features: (i) growth, (ii) metabolic and anabolic activity, and (iii) deployment of specific stress-adaptive responses. Though the

schematic in Figure 13 presents these as discrete states, it may be more realistic to think of them as stations along continua. The susceptibility of a cell will depend on both the physiological state and the particular antimicrobial agent. Here are a few examples to illustrate the diversity of protected states.

In general, when biofilm microorganisms are compared to planktonic cells for antimicrobial susceptibility, the comparison is to a growing batch culture (Fig. 13A). These are cells that may be relatively sensitive to antimicrobial attack because their current environment is growth permissive and their current investment is in cell growth and replication rather than survival. Cells that transition to a nongrowing but still active state (Fig. 13B) may quickly acquire tolerance to some agents. In the conceptualization of Figure 13, this state is conceived of as cells with an active membrane potential and capacity for generation of some ATP along with sub-maximal capacity for transcription and translation. These cells do not exhibit DNA replication, cell wall synthesis, or balanced translation of all of the proteins required to make a new cell. In such a state, bacteria become insensitive to β-lactam antibiotics, which lyse cells by inhibiting cell wall synthesis as the cell continues to expand (16). Cells that transition to the inactive, nongrowing state (Fig. 13C) lack any catabolism or anabolism. Such a cell cannot maintain a membrane potential and thus may become insensitive to aminoglycoside antibiotics, which depend on active transport to reach their intracellular targets (17). The dormant state (Fig. 13D) is conceived of as distinct from the inactive, nongrowing state (Fig. 13C).

The dormant state is also metabolically inactive and nongrowing. To enter the dormant state, however, the cell has implemented protective modifications. Such modifications could include, hypothetically, alteration of membrane lipid and porin composition to reduce permeability, hibernation of ribosomes, inhibition of transcription and replication machinery, and deployment of enzymes that protect against oxidative stress without consuming ATP (e.g., catalase). In contrast, the nongrowing, inactive state (Fig. 13C) is an energetically disabled cell that has no other protective modifications. By way of an analogy, the state in Figure 13C could be compared to a car that has run out of gas by the side of the road and been abandoned, whereas a vehicle analogous to the cell state in Figure 13D, while also out of gas, has had the windows rolled up, the radiator drained, the battery disconnected, and a cover tied over it. Such a dormant cell state could confer tolerance to a wide variety of antimicrobial challenges. The much-discussed persister cell may represent such a dormant state (18, 19). Metabolically active bacteria are able to sense their environment and actively respond to the presence of an antimicrobial stress. In the schema of Figure 13, either growing cells (Fig. 13A) or nongrowing yet active cells (Fig. 13B) have the capacity to deploy active stress responses (resulting in the states shown in Figs. 13E and 13F, respectively). Examples of stress responses that have been demonstrated in bacterial bio-films include catalase induction upon treatment with hydrogen peroxide (20), β-lactamase induction upon treatment with imipenem (21), and induction of the lipopolysaccharide-modifying *pmr* operon upon treatment with colistin (22). In each of these examples, the induced gene or genes enhance the capacity of the bio-film to tolerate the antimicrobial either by augmenting destruction of the antimicrobial agent or by modifying the cell to make it less susceptible.

Because biofilms are known to contain niches of varying environmental chemistry and biological activity, it is important to recognize that a biofilm could harbor cells in more than one, possibly all, of the states shown in Figure 13 (23). This physiological heterogeneity or diversification is likely an important factor in the tolerance of the biofilm state. Note that none of these states is necessarily exclusively associated with either a planktonic or biofilm cell.

One difficulty with analyzing the physiological variety diagrammed in Figure 13 is a lack of standard quantitative measures of most of the physiological characteristics. There is an excellent quantitative parameter to characterize microbial growth: specific growth rate. Techniques to measure local growth rates within bio-films could offer insight into the spectrum of physiological states that influence antimicrobial susceptibility. In addition, it would be helpful to have quantitative measures of the overall cellular capacity for transcription or translation, the relative expression of adaptive stress responses, and some quantitative definition of dormancy.

The cell states diagrammed in Figure 13 are surely associated with the differential expression of specific sets of genes in a particular organism. One issue to keep in mind in interpreting the analysis of antimicrobial susceptibility of genetic mutants grown as biofilms is that a mutation that affects the areal cell density of the biofilm could indirectly alter its susceptibility. Indeed, this effect is to be expected, as discussed above and presented in Figures 3 to 5. Some of the systems that have been reported to contribute to biofilm antimicrobial tolerance include the stringent response (24), the SOS response (25), efflux pumps (26, 27), quorum sensing (28), toxin-antitoxin modules (29, 30), the elaboration of periplasmic or extracellular polysaccharides (31, 32, 33), and others (34–36). At this time it is still too early to be able to identify a consensus genetic basis for biofilm antimicrobial tolerance, but these details are certain to follow.

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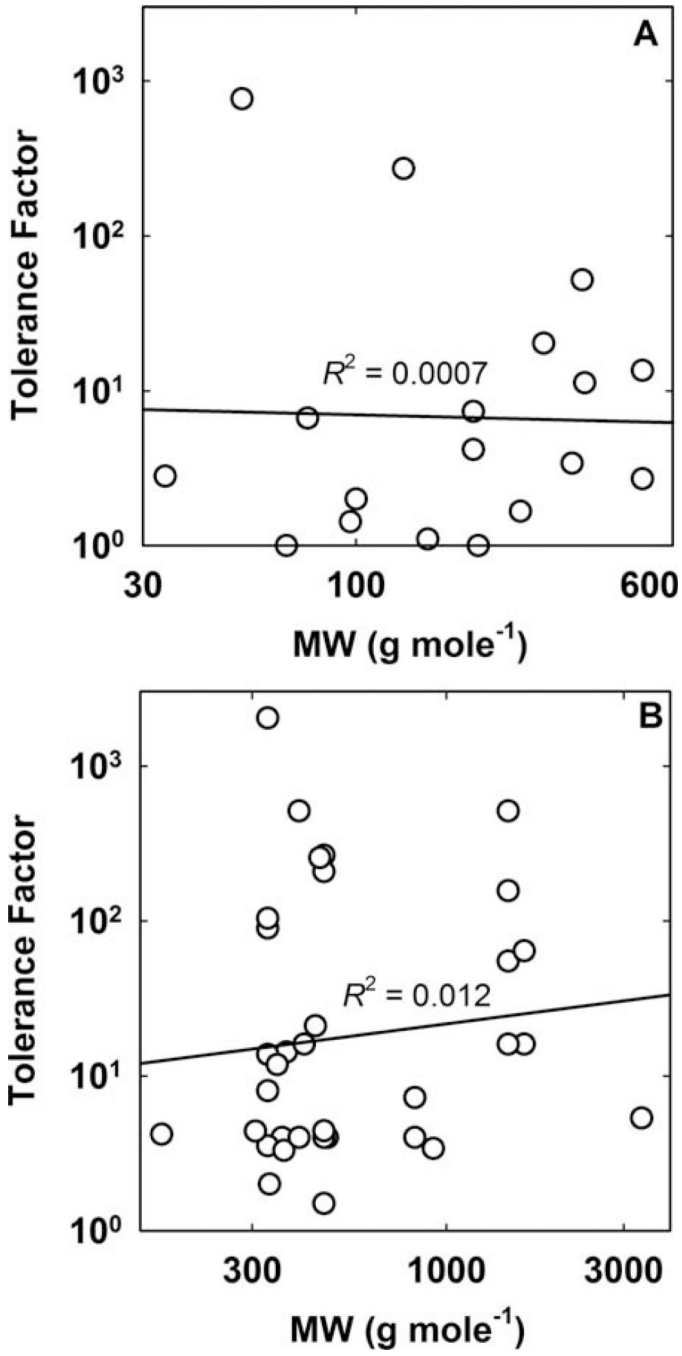

FIGURE 1.

Tolerance factors versus antimicrobial agent molecular weight for the data on (A) biocides and antiseptics from Table 1 and (B) antibiotics from Table 2. doi:10.1128/microbiolspec.MB-0010-2014.f1

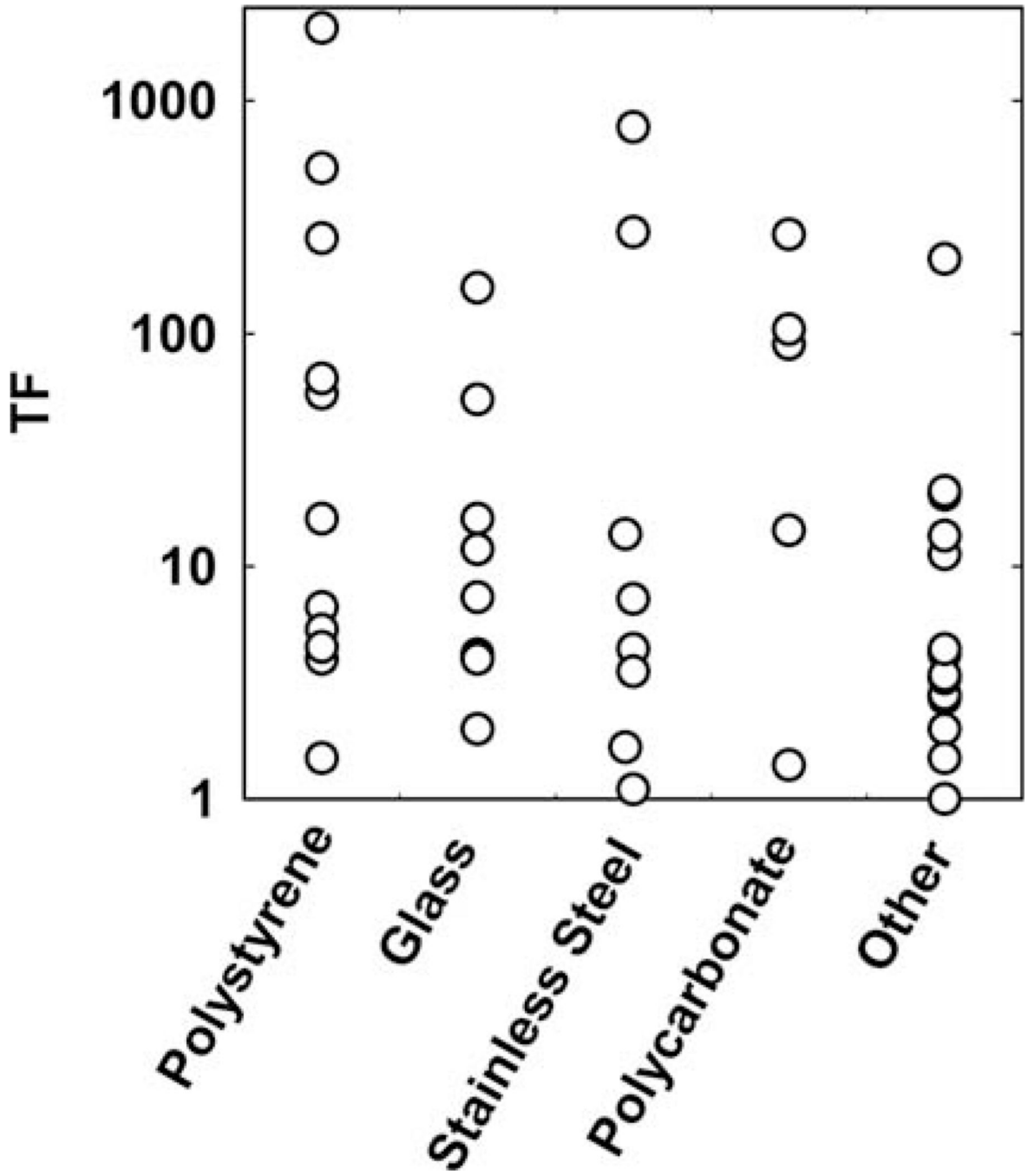

FIGURE 2.
Tolerance factors grouped and compared by substratum material. doi:10.1128/microbiolspec.MB-0010-2014.f2

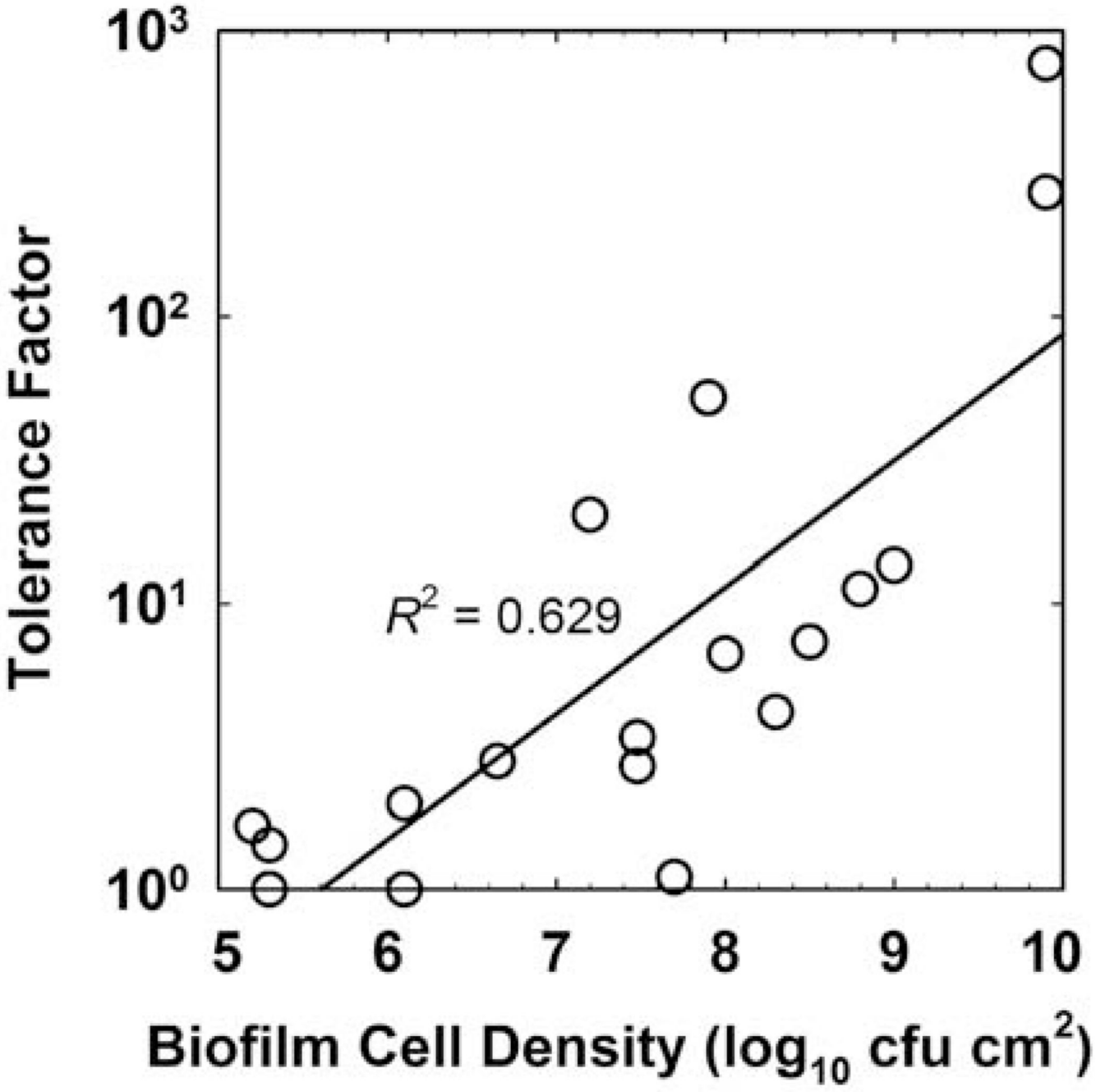

FIGURE 3.
Tolerance factor versus biofilm cell density for the data in Table 1. The line is the least squares regressed fit. doi:10.1128/microbiolspec.MB-0010-2014.f3

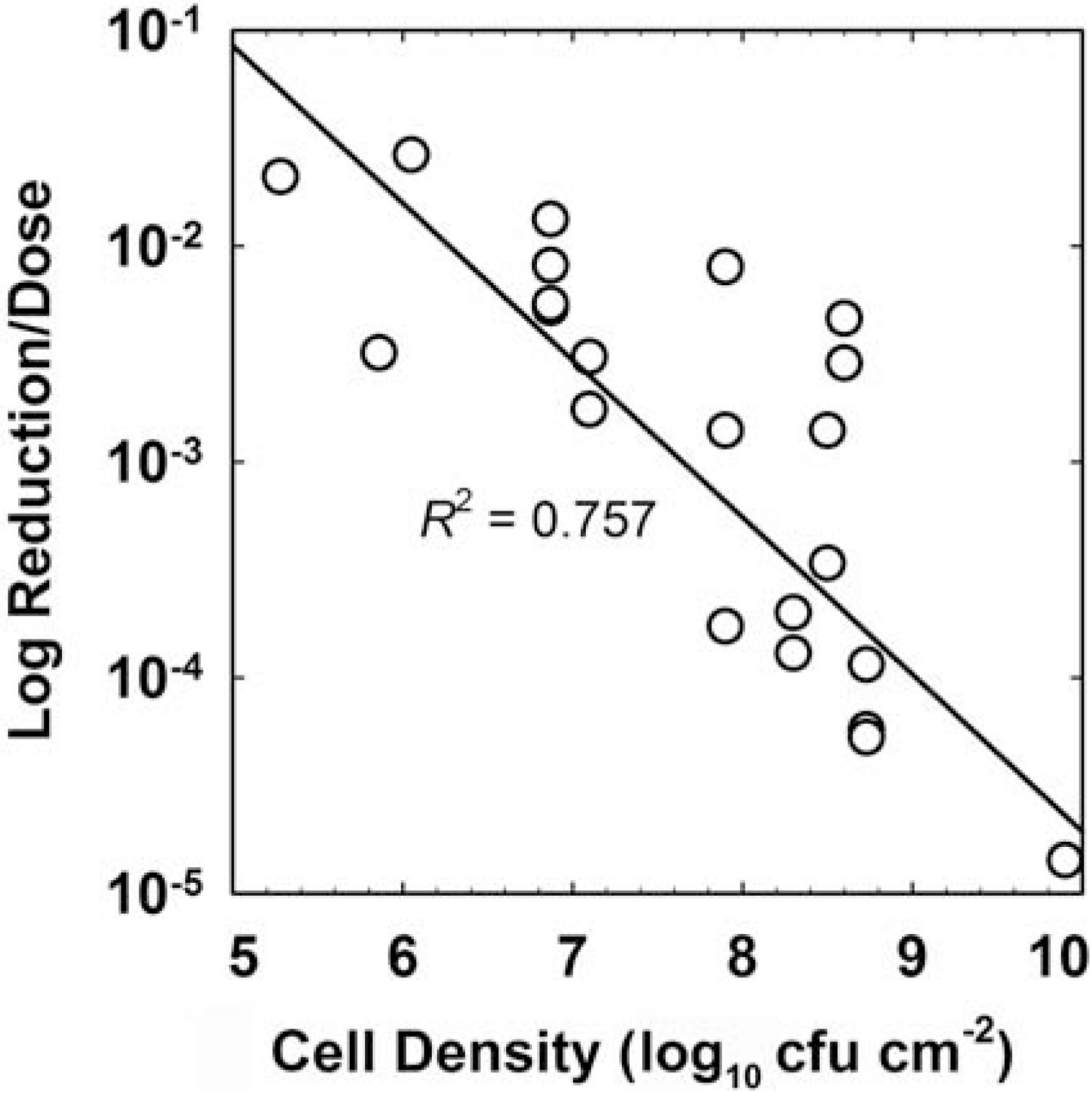

FIGURE 4.

Efficacy of chlorine treatment against biofilms as a function of the untreated control biofilm areal cell density. The y-axis is the reported log reduction divided by the product of dose concentration and duration ($C_B t_B$). The line is the least squares regressed fit. Sources: references 8, 37–44. doi:10.1128/microbiolspec.MB-0010-2014.f4

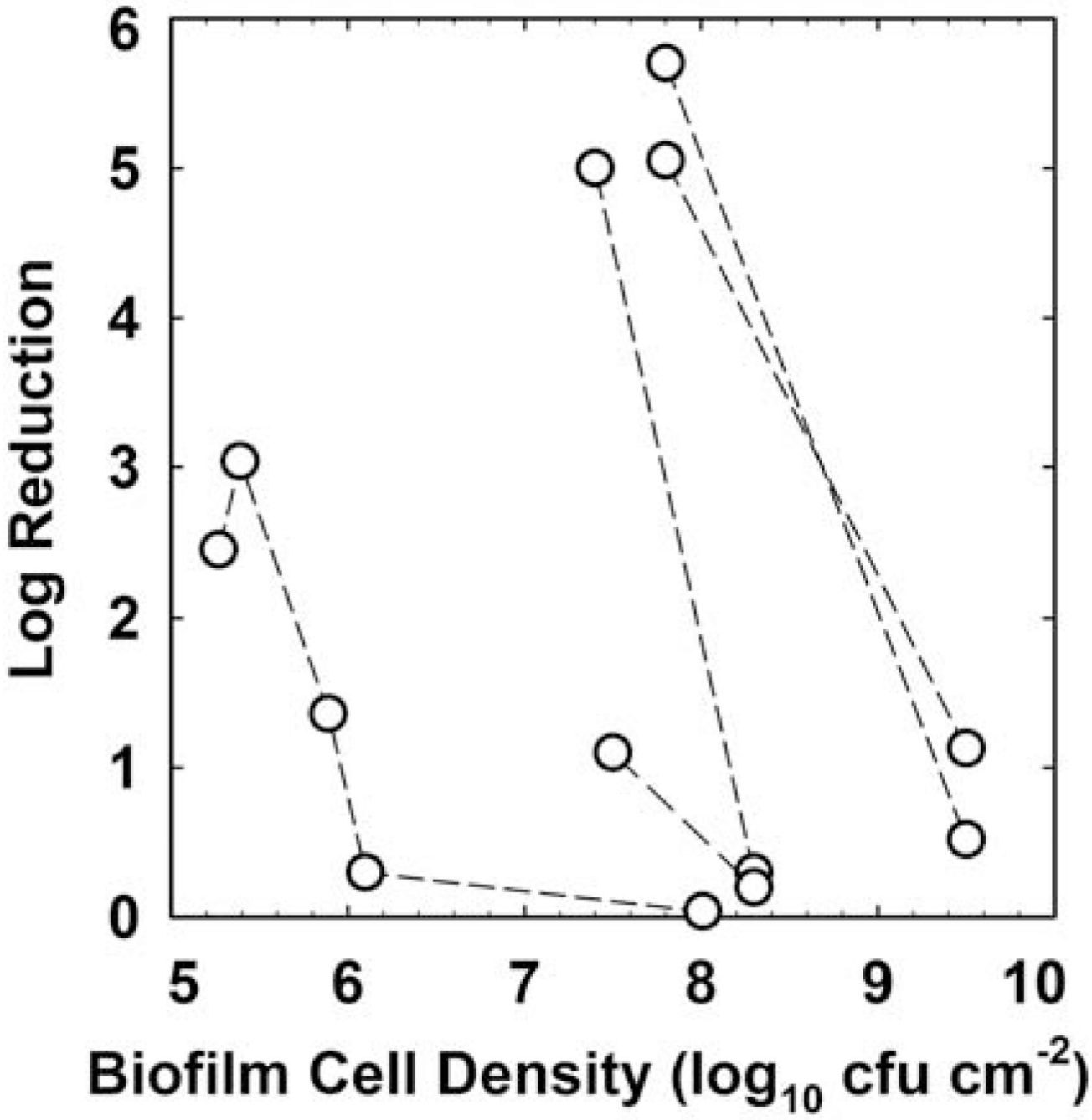

FIGURE 5.

Antibiotic efficacy against *Pseudomonas aeruginosa* biofilms as a function of the untreated control biofilm areal cell density. Dashed lines connect data points from the same investigation. The antibiotics used include tobramycin, cipro-floxacin, and gentamicin. Sources: references 11, 45–47. doi:10.1128/microbiolspec.MB-0010-2014.f5

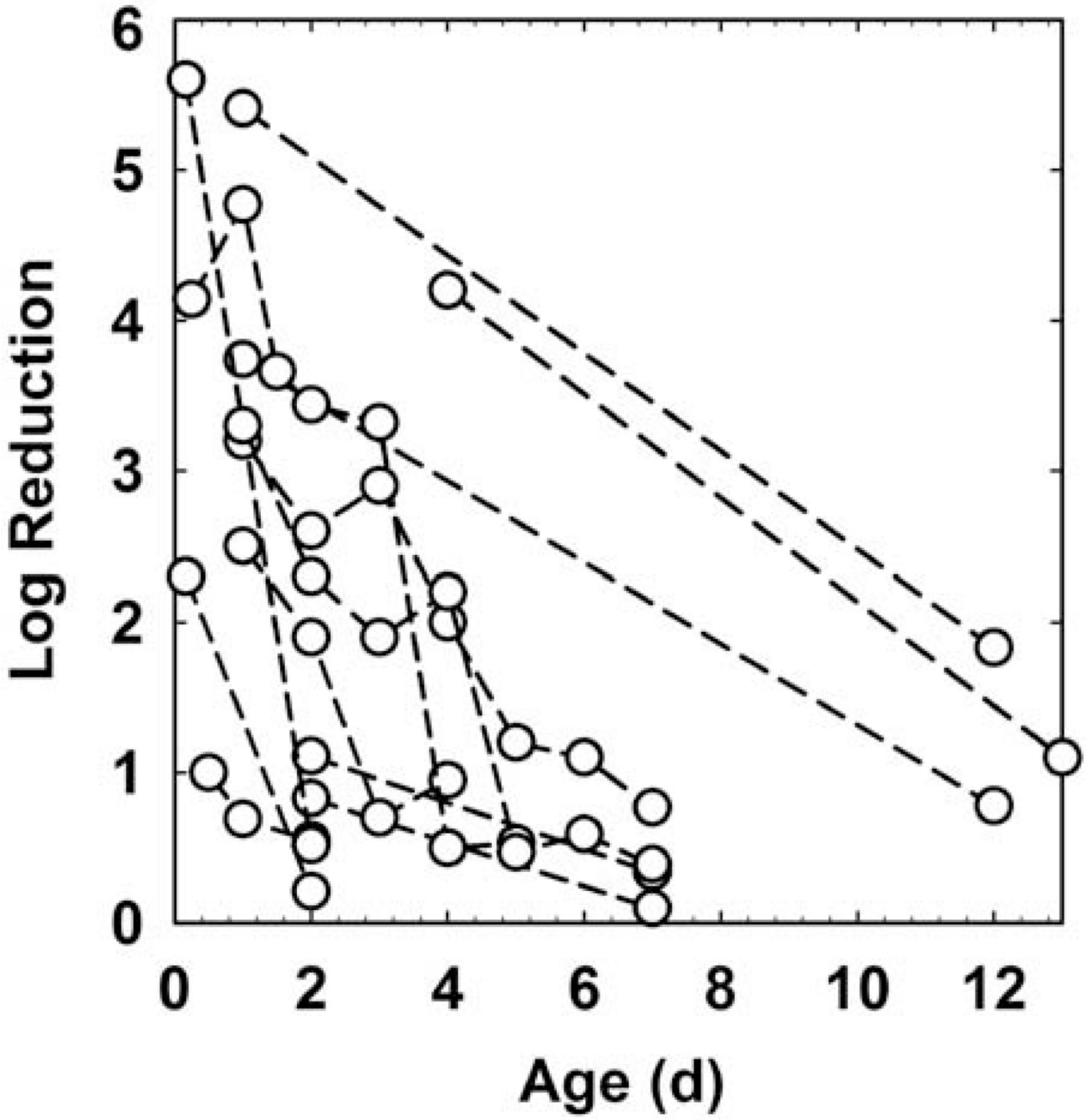

FIGURE 6.

Antimicrobial efficacy as a function of biofilm age. Dashed lines connect data points from the same investigation. Sources: references 11, 25, 45, 47, 48–51. doi:10.1128/microbiolspec.MB-0010-2014.f6

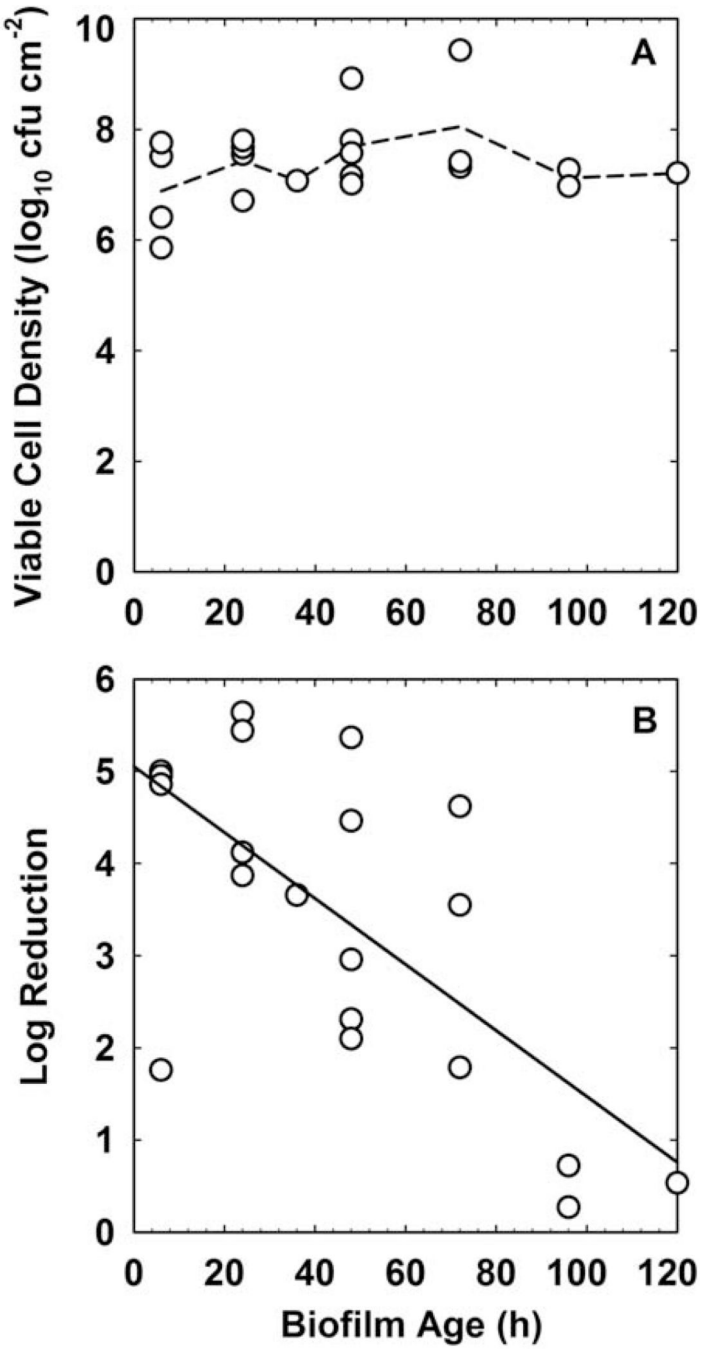

FIGURE 7.

(A) Maturation of *S. aureus* biofilm and (B) change in gentamicin susceptibility with age. The dashed line in panel A connects the mean values at each time point. The solid line in panel B is the least squares regressed fit. Source: reference 11. doi:10.1128/microbiolspec.MB-0010-2014.f7

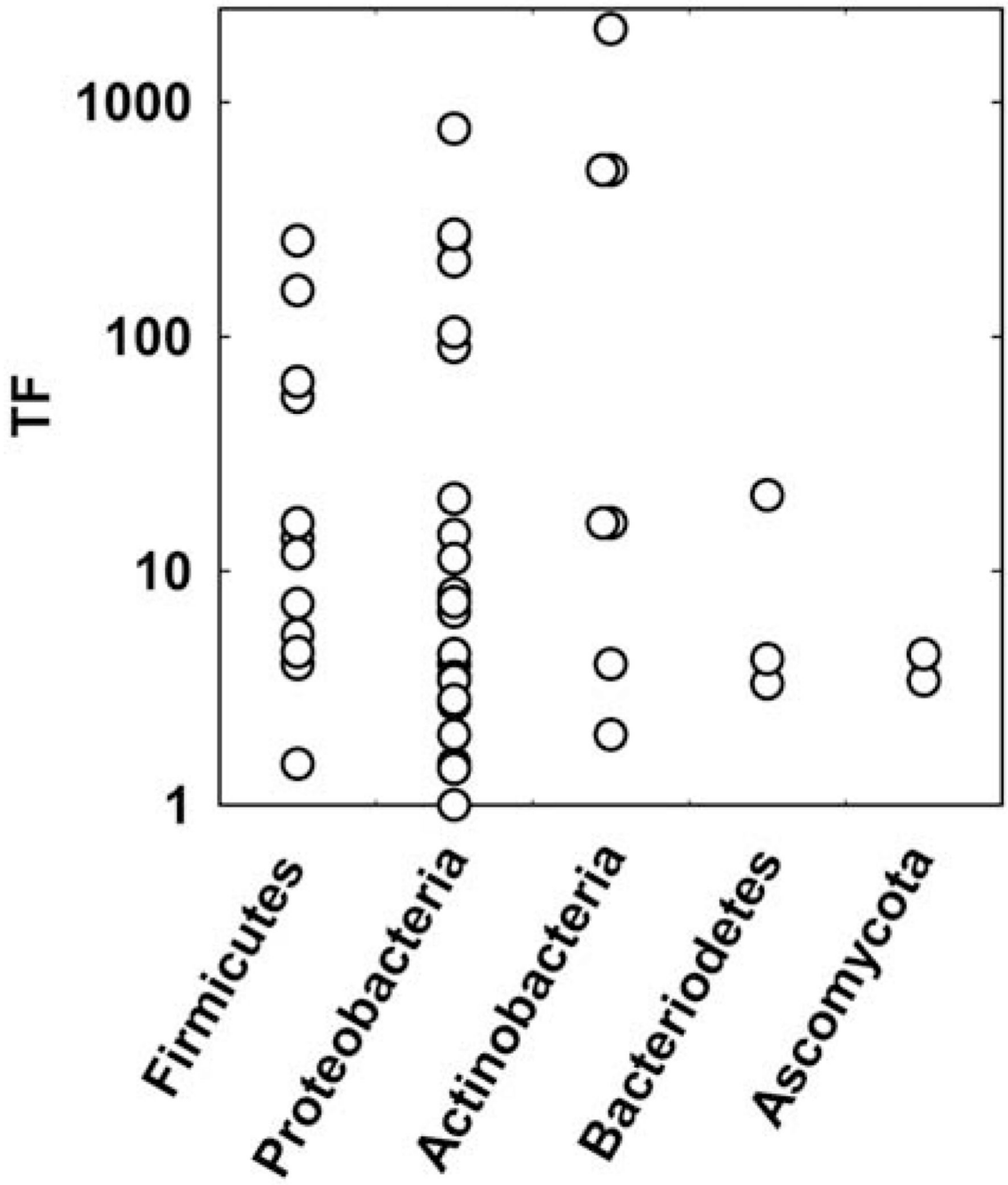

FIGURE 8.
Tolerance factors for biocides and antibiotics for four bacterial phyla and a fungus. doi: 10.1128/microbiolspec.MB-0010-2014.f8

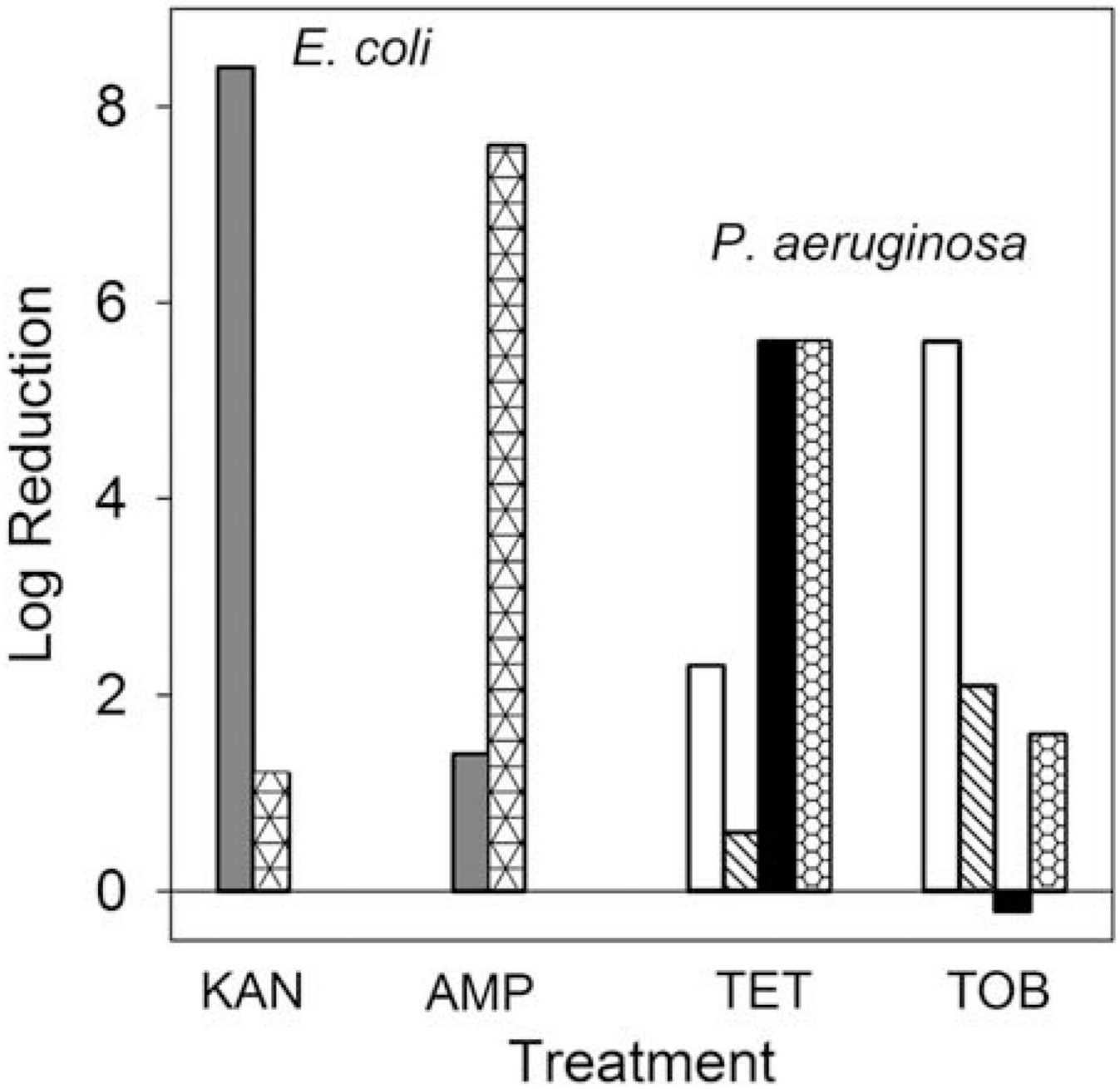

FIGURE 9.

Medium effects on biofilm susceptibility to antibiotics. The different bar fills denote various media: LB (gray); LB + glucose (triangles); TSA, aerobic (white); TSA, anaerobic (hatched); noble agar, aerobic (black); noble agar, anaerobic (honeycomb). Sources: reference 52 for E. coli and unpublished data of Borriello and Stewart for P. aeruginosa. doi: 10.1128/microbiolspec.MB-0010-2014.f9

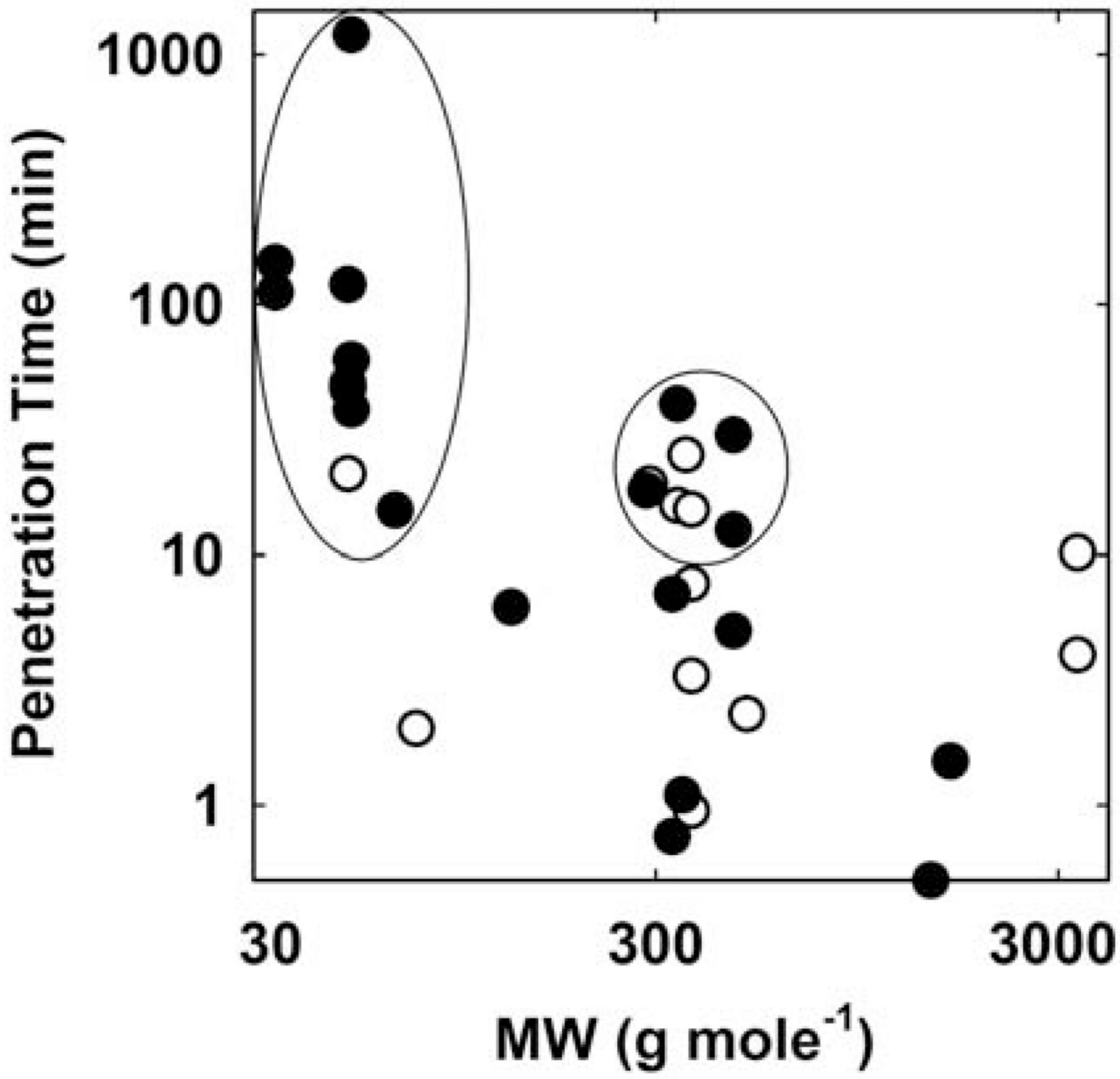

FIGURE 10.

Experimentally measured antimicrobial penetration times in biofilms versus molecular weight of the antimicrobial. The penetration time was determined as the time to attain, at the base or center of the biofilm, 50% of the equilibrium concentration of the antimicrobial agent either through a direct measurement of the antimicrobial agent (solid circles) or by loss of membrane integrity detected with a fluorescent probe (open circles). Penetration times greater than 12 min are circled. Sources: references 15, 37, 53–63. doi:10.1128/microbiolspec.MB-0010-2014.f10

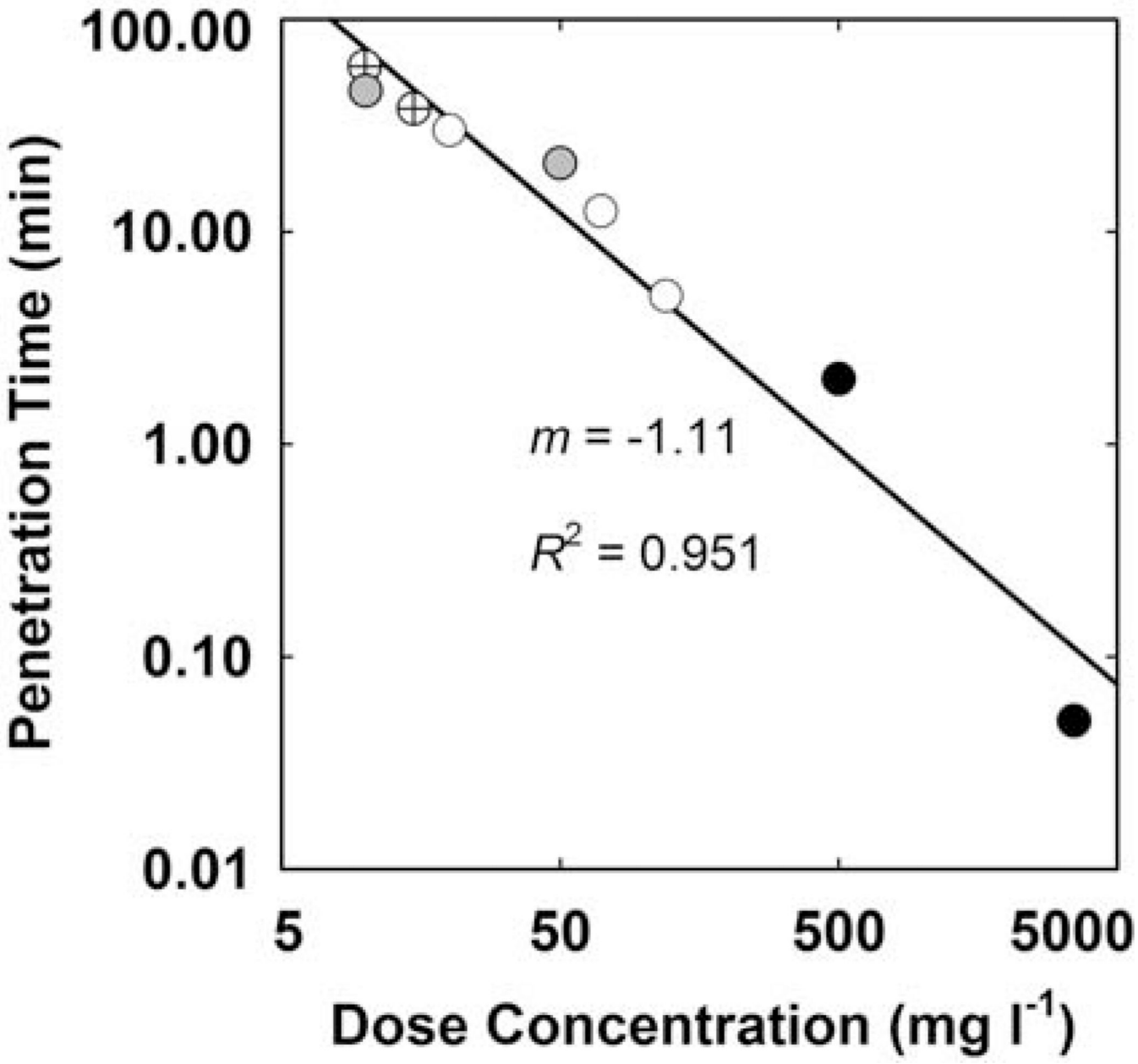

FIGURE 11.

Experimentally measured antimicrobial penetration times in biofilms versus dose concentration. The line is the least squares regressed fit. Symbols indicate data for chlorine (cross, 55), chlorine (gray, 54), tobramycin (white, 62), peracetic acid (black, 53). doi: 10.1128/microbiolspec.MB-0010-2014.f11

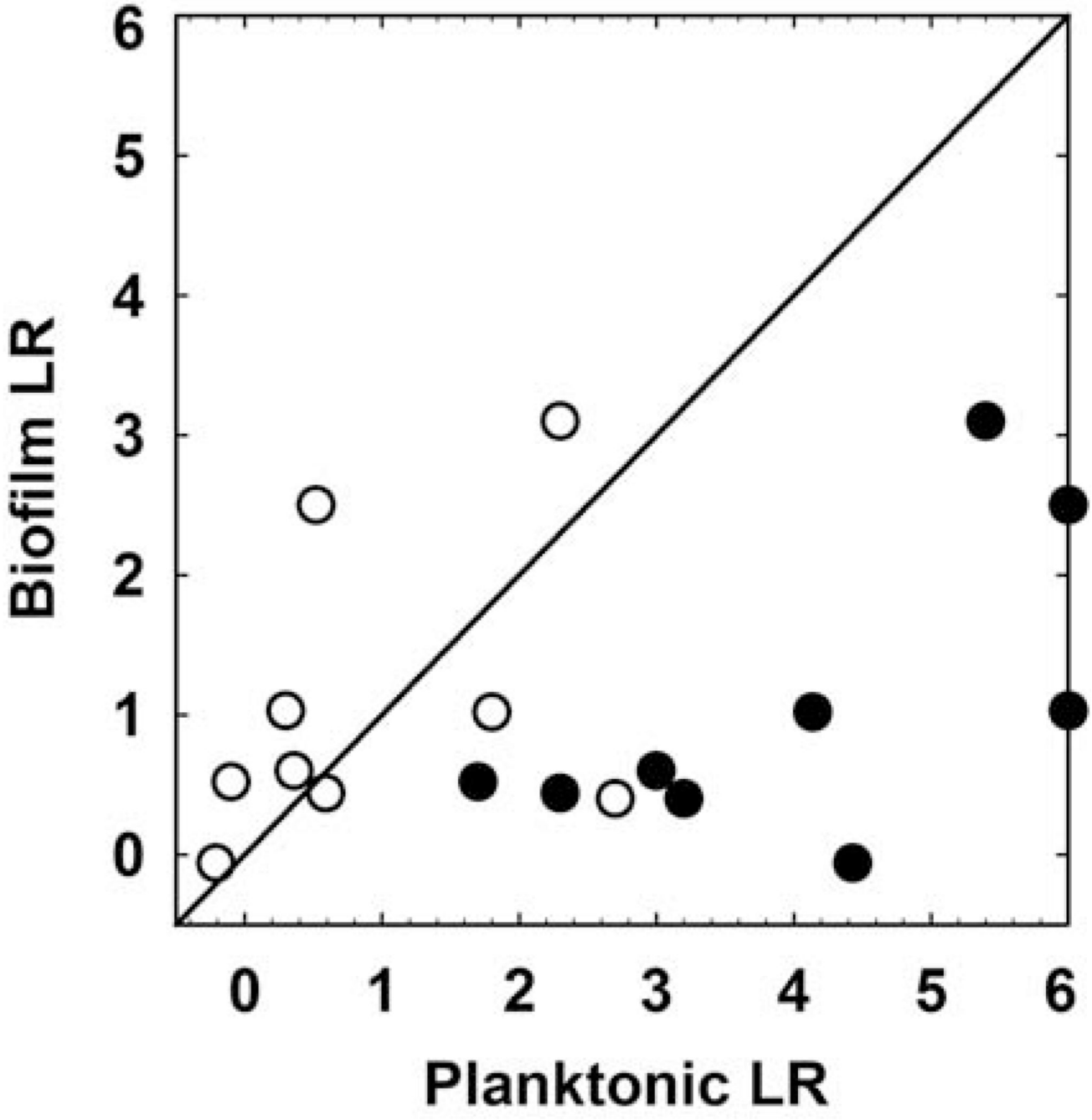

FIGURE 12.

Comparison of antimicrobial susceptibility of exponential phase planktonic (solid symbols) or stationary phase planktonic (open symbols) to biofilm cells. The solid line is the line of equality. Points below the line indicate that biofilm cells were less susceptible than planktonic cells. Points above the line indicate that planktonic cells were less susceptible than biofilm cells. Sources: references 38, 64–66. doi:10.1128/microbiolspec.MB-0010-2014.f12

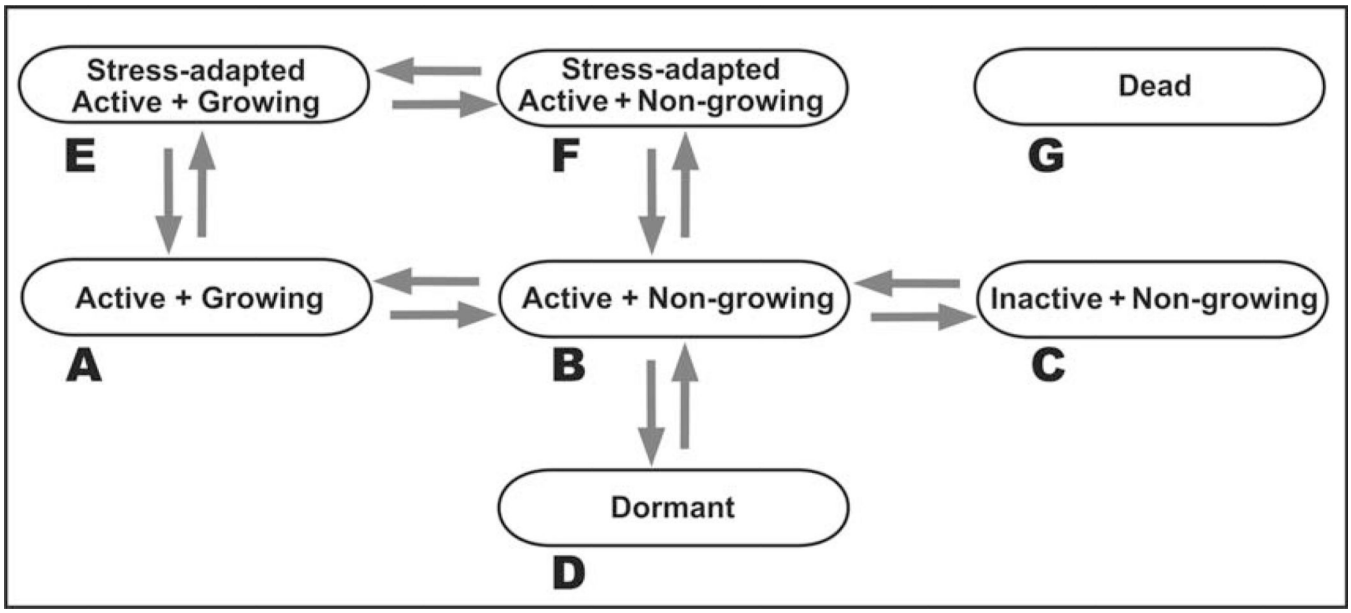

FIGURE 13.

Conceptual diagram of distinct cell states important for antimicrobial sensitivity. The dead cell state can presumably be accessed from any of the other states. doi:10.1128/microbiolspec.MB-0010-2014.f13

TABLE 1

Selected examples of tolerance of bacteria in biofilms to biocides and antiseptics

Organisms	Agent	Molecular weight(g mole^{-1})	Substratum[a]	($\log_{10}$ cfu cm^{-2})X_o	*TF*	References
Pseudomonas aeruginosa,	Hypochlorite, pH 11	52.5	SS	9.9	767	37
Klebsiella pneumoniae	Chlorosulfamate	131			272	
P. aeruginosa	Peracetic acid	76.1	PS	8	6.7	67
Aggregatibacter	Chlorhexidine	506	CN	7.48	2.7	68
actinomycetocomitans	Cetylpyridinium chloride	340			3.4	
Legionella in mixed species	Glutaraldehyde	100	RR	6.1	2.0	69
	Bromo-nitropropane-diol	200			1.0	
P. aeruginosa	Hydrogen peroxide	34	PE	6.65	2.8	70
Mixed drinking water	Chlorine dioxide	67.5	G	5.3	1.0	71
Staphylococcus aureus	Benzalkonium chloride	360	G	7.9	52	38
P. aeruginosa	Bromine	96.9	PC	5.3	1.4	72
P. aeruginosa	Benzylchlorophenol,	195	G	8.5	7.4	39
S. aureus	phenylphenol				4.2	
Salmonella typhimurium	Triclosan	290	Pellicle	7.2	20	73
Citrobacter diversus	Povidone-iodine	365	S	8.8	11	74
Listeria monocytogenes	Iodine	254	SS	5.2	1.7	8
Mixed paper mill white water	Thymol	150	SS	7.7	1.1	75
Mixed oral	Chlorhexidine	506	HAP	9	13.5	76

[a] Abbreviations: SS, stainless steel; PS, polystyrene; CN, cellulose nitrate; RR, red rubber; PE, polyester; G, glass; PC, polycarbonate; S, silicone; BR, Buna rubber; HAP, hydroxyapatite

TABLE 2

Selected examples of tolerance of bacteria or fungi in biofilms to antibiotics

Organisms	Agent	Molecular weight(g mole^{-1})	Substratum[a]	($\log_{10}$ cfu cm^{-2})X_o	*TF*	References
Propionibacterium acnes	Rifampin	823	G		4	77
	Daptomycin	1620			16	
	Vancomycin	1468			16	
	Penicillin G	334			2	
Corynebacterium urealyticum	Ciprofloxacin	330	PS		2048	78
	Moxifloxacin	401			512	
	Vancomycin	1468			512	
Pseudomonas aeruginosa	Gentamicin	478			4	79
	Tobramycin	468			4	
	Ciprofloxacin	330			8	
	Ofloxacin	361			4	
P. aeruginosa	Tobramycin	468	SS	9.6	4.4	80
	Ciprofloxacin	330			3.5	
K. pneumoniae	Ciprofloxacin	330	PC	10.3	90	64
	Ampicillin	371		10.2	14	
Staphylococcus epidermidis	Ciprofloxacin	330	SS	8.9	14	81
	Rifampin	823			7	
P. aeruginosa	Tobramycin	468	PC	10.4	265	82
	Ciprofloxacin	330			104	
P. aeruginosa	Tobramycin	468	S	8.3	208	45
				7.4	1.5	
S. aureus	Nisin	3354	PS	7.5	5.3	83
	Vancomycin	1468		7.8	55	
S. epidermidis	Levofloxacin	350	G	10.3	12	48
	Vancomycin	1468			157	
Porphyromonas gingivalis	Amoxicillin	365	CA		3.3	84
	Doxycycline	444			21	
	Metronidazole	171			4.2	

Organisms	Agent	Molecular weight(g mole^{-1})	Substratum[a]	($\log_{10}$ cfu cm^{-2})X_o	*TF*	References
Staphylococcus lugdunensis	Cefazolin	456	PS		256	85
	Rifampin	823			4	
	Daptomycin	1620			64	
	Moxifloxacin	401			4	
	Naficillin	414			16	
Candida albicans	Amphotericin B	923	PVC		3.4	86
C. albicans	Fluconazole	306	PVC		4.4	87

[a] Abbreviations: as for Table 1; CA, cellulose acetate; PVC, polyvinyl choride