The influence of heteroresistance on minimum inhibitory concentration (MIC), investigated using weak-acid stress in food spoilage yeasts

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Abstract

Populations of microbial cells may resist environmental stress by maintaining a high population-median resistance (IC_{50}) or, potentially, a high variability in resistance between individual cells (heteroresistance); where heteroresistance would allow certain cells to resist high stress, provided the population was sufficiently large to include resistant cells. This study sets out to test the hypothesis that both IC_{50} and heteroresistance may contribute to conventional minimal-inhibitory-concentration (MIC) determinations, using the example of spoilage-yeast resistance to the preservative sorbic acid. Across a panel of 26 diverse yeast species, both heteroresistance and particularly IC_{50} were positively correlated with predicted MIC. A focused panel of 29 different isolates of a particular spoilage yeast was also examined (isolates previously recorded as *Zygosaccharomyces bailii*, but genome resequencing revealing that several were in fact hybrid species*, Z. parabailii and Z. pseudobailii*). Applying a novel high-throughput assay for heteroresistance, it was found that IC_{50} but not heteroresistance was positively correlated with predicted MIC when considered across all isolates of this panel, but the heteroresistance-MIC interaction differed for the individual *Zygosaccharomyces* subspecies. *Z. pseudobailii* exhibited higher heteroresistance than *Z. parabailii* whereas the reverse was true for IC_{50}, suggesting possible alternative strategies for achieving high MIC between subspecies. This work highlights the limitations of conventional MIC measurements due to the effect of heteroresistance in certain organisms, as the measured resistance can vary markedly with population (inoculum) size.

Importance

Food spoilage by fungi is a leading cause of food waste, with specialised food spoilage yeasts capable of growth at preservative concentrations above the legal limit, in part due to heteroresistance allowing small subpopulations of cells to exhibit extreme preservative resistance. Whereas

heteroresistance has been characterised in numerous ecological contexts, measuring this phenotype systematically and assessing its importance are not encompassed by conventional assay methods. The development here of a high-throughput method for measuring heteroresistance, amenable to automation, addresses this issue and has enabled characterisation of the contribution that heteroresistance may make to conventional MIC measurements. We used the example of sorbic acid heteroresistance in spoilage yeasts like *Zygosaccharomyces* spp, but the approach is relevant to other fungi and other inhibitors, including antifungals. The work shows how median resistance, heteroresistance and inoculum size should all be considered when selecting appropriate inhibitor doses in real-world antimicrobial applications such as food preservation.

Introduction

Resistance of microorganisms to inhibitory agents is a growing problem in therapeutics (Andersson et al., 2019, Dewachter et al., 2019) and food supply chains (Davies et al. 2020). The resistance of a microorganism to a stressor is typically described using a conventional minimum inhibitory concentration (MIC) measurement, which reflects the concentration of a stressor required to prevent growth at a particular fixed inoculum size (Box 1). Other measures of stress resistance include IC_{50}, a population-median measure describing the concentration required to inhibit either 50% of cells in a population (as used in the present work, Box 1) or all cells by 50%, depending on the study. In recent years there has been growing recognition also of heteroresistance, describing the heterogeneity of resistance between individual clonal cells (Box 1), which appears to be very ubiquitous (Bishop et al. 2007, Holland et al. 2014, Jing et al. 2018, Levy et al., 2012). However, the extent to which differences in heteroresistance may contribute to MIC of a cell inoculum remains barely explored, especially relative to traditional measures such as IC_{50}. The present work aimed to address this gap in our knowledge, as it could explain variability in MIC data that are used as common standards, due to parameters such as population size and the inoculum effect (Box 1).

Box 1: Definitions of parameters

1) **MIC:** minimum inhibitory concentration, i.e., lowest concentration of a stressor required to completely inhibit the growth of an inoculum of a microorganism.
2) **MIC^{EXP}:** Experimentally derived MIC, at a given inoculum size, e.g., the MIC^{EXP} for 100 cells of a given strain could be different to the MIC^{EXP} for 10,000 cells of the same strain.
3) **MIC^{MODEL}:** MIC of 10,000 cells/well predicted using a lognormal distribution curve, fitted to MIC^{EXP} values (see *Fitting to normal and lognormal distributions*)
4) **Heteroresistance:** Cell-cell phenotypic heterogeneity in a resistance phenotype. Here quantified as the standard deviation of a lognormal distribution of single cell resistances
5) **IC_{50}:** Population-median resistance, here defined as the mean of a lognormal distribution of single cell resistances
6) **Inoculum effect:** Phenomenon by which MIC^{EXP} increases with inoculum size, due to increased probability of resistant cells being present

Phenotypic heterogeneity among individual cells of genetically uniform populations is considered an evolutionarily selected strategy akin to bet hedging (Ackermann 2015). It describes cell-to-cell variation in a phenotype (e.g. stress resistance) within a cell population, resulting in phenotypically (not genotypically-) distinct subpopulations among which some may be better equipped to thrive if conditions change (Ackermann 2015, Smith et al., 2007, Levy et al. 2012, Stratford et al 2019). The rationale is that generation of phenotypic heterogeneity, may be a relatively low cost strategy (metabolically) for improving survival-chances in uncertain conditions. Such heterogeneity in the case of stress resistance can be observed in almost any resistance phenotype: examples of heteroresistance are evident in microbial responses to antibiotics (Dewachter et al., 2019, Scheler et al., 2020, Jing et al. 2018), heat (Levy et al., 2012), osmotic (Stratford et al. 2019), metal (Holland et al., 2014) and weak organic acid (Stratford et al. 2014, Geoghegan et al. 2020) stresses. Mechanistic determinants of heteroresistance have been characterised, including cell-to-cell variation in gene expression linked to particular 'high' or 'low'-variation promoters or transcription factors, commonly referred to as gene expression noise (Silander et al. 2011, Urchueguia et al., 2021, Newman et al. 2006). Housekeeping genes tend to be expressed with low variation between cells, whereas high-variation promoters are more commonly associated with stress-response genes (Newman et al. 2006). Engineering increased variation of expression of an oxidative stress response gene has been shown to increase resistance to oxidative stress in *Saccharomyces cerevisiae* (Liu et al., 2018), while dampening of expression variation in similar functions gave decreased resistance (Smith et al., 2007).

As food spoilage microorganisms can exhibit both high population-median resistance and heteroresistance to food preservatives (Stratford et al. 2014), they provide a good example with which to address this study's main aim to characterise the relationship between MIC and heteroresistance versus population-median resistance. Among the major food preservatives, sorbic acid is a weak organic acid preservative widely used in condiments and drinks of pH ≤4, conditions in which the acid is undissociated and capable of traversing the plasma membrane. Several modes of

101 action have been proposed for sorbic acid, including cytosolic acidification (Stratford et al., 2013a)
102 and disruption of the mitochondrial electron transport chain (Stratford et al., 2020). Efficacy of
103 sorbic acid as a food preservative is hampered by the intrinsic resistance of specialised spoilage
104 yeasts, which are capable of growth at sorbic concentrations exceeding the maximum legal limit
105 dictated by food standards agencies (Stratford et al., 2013b).

106 *Zygosaccharomyces bailii* is a food spoilage yeast exhibiting a high level of resistance to weak organic
107 acids (Stratford et al. 2014). Two interspecies hybrids related to *Z. bailii* and also reported to spoil
108 preserved foods are *Z. parabailii* and *Z. pseudobailii,* formed through hybridisation events between
109 ancestral *Z. bailii* and two unknown *Zygosaccharomyces* species, sharing approximately 90-93%
110 sequence identity with *Z. bailii* (Braun-Galleani et al., 2018). *Z. bailii* has several proposed
111 mechanisms of resistance to weak organic acids, including a relatively thick and rigid plasma
112 membrane (Lindberg et al. 2013, Lindahl et al. 2016), metabolism of sorbic acid to less inhibitory
113 products (Mollapour and Piper, 2001), metabolism that is primarily fermentative (Stratford et al.
114 2020) and a low cytosolic pH (Stratford et al. 2013b, 2014). In addition to being relatively resistant to
115 sorbic acid (according to MIC or IC_{50}), *Z. bailii* also shows high sorbic acid heteroresistance (Stratford
116 et al. 2013b), with a small proportion of cells capable of resisting extreme concentrations even
117 though they may take several weeks to grow to detectable levels.

118 The food-industry relevance of *Z. bailii*, coupled with its high heteroresistance makes it an ideal
119 model for studying the relative contributions of population-median resistance and heteroresistance
120 to MIC. In this study we assess and compare these parameters in a panel of diverse spoilage yeasts
121 as well as more select panels of *Z. bailii, Z. parabailii* and *Z. pseudobailii* isolates. The work highlights
122 the importance of context when assessing the resistance of a microorganism, with population-
123 median resistance, heteroresistance and inoculum size impacting the resistance of a given inoculum,
124 nuances which are lost when an MIC at a single inoculum size is taken.

125

Materials and Methods

Yeast species and strains

Yeast species and strains used for experiments in this study are listed in Tables 1, 2 (alternative identifiers and origin of isolation are given where available). Members of a large, initial *Zygosaccharomyces* panel (Table S1) were designated *Z. bailii*, *Z. parabailii* or *Z. parabailii* based on whole genome sequencing (WGS) analysis (see Genome sequencing) and a subset of these comprised the main *Zygosaccharomyces* panel used for experiments (Table 1). Several species previously designated *Z. bailii* were redesignated *Z. parabailii* or *Z. pseudobailii* based on the WGS analysis (below). Members of a wide panel of isolates from more diverse yeast genera (Table S2) were identified previously based on sequencing of the D1/D2 region of the 25S ribosomal DNA (rDNA) (Stratford et al. 2013b).

Culture conditions

The YEPD growth medium used for culturing [20 g/l glucose, 20 g/l bacteriological peptone (Oxoid), and 10 g/l yeast extract (Oxoid)] was adjusted to pH 4 using 5 M HCl prior to sterilisation by autoclaving. Yeasts were stored at –80°C in cryovials in YEPD medium mixed with 0.2 volumes glycerol. Cells were grown from frozen on YEPD supplemented with 2% agar (Oxoid) for 3 days at 30°C, before maintenance at 4°C for up to 3 weeks. For each biological replicate in experiments, a single colony was suspended in 1 ml YEPD broth and, after determination of OD_{600}, an aliquot transferred to 10 ml YEPD broth in order to give a starting OD_{600}~0.02. These cultures were incubated in 50 ml Erlenmeyer flasks at 24°C for >12 hours with shaking (120 rev/min) prior to use in experiments at OD_{600} 0.1 – 2.0, when cells were growing in exponential phase. Desired concentrations of sorbic acid in the growth medium were produced using a stock solution of 20 mM potassium sorbate (Sigma-Aldrich) in YEPD, adjusted to pH 4 using 5 M HCl. Final concentrations of sorbic acid in YEPD agar or broth were produced by mixing appropriate ratios of YEPD medium (pH

4) with the 20 mM sorbic acid-supplemented medium. All steps were carried out under aseptic conditions.

De novo genome sequencing

De novo genome sequencing was carried out by KeyGene (Wageningen, Netherlands) by mapping short Illumina reads to long PacBio reads for *Z. bailii* (7846), *Z. parabailii* (3699, containing *Z. bailii*-derived 'A' genome and hybrid 'B' genome) and *Z. pseudobailii* (3697, containing *Z. bailii*-derived 'A' genome and hybrid 'C' genome). Yeast isolates were grown in YEPD medium at 25°C for 48 hours. Subsequently, cells were harvested by centrifugation (3000 *g*, 3 min), the supernatant removed and the pelleted cultures freeze dried prior to DNA isolation. Isolation of high molecular weight DNA suitable for long-read sequencing was carried out by Keygene based on methods described previously (Zhang et al. 2012, Datema et al. 2016).

To generate the sequencing data for *Z. pseudobailii* and *Z. parabailii*, one PacBio SMRT cell (Sequel) was used for each isolate. The same DNA was used for generating paired-end Illumina reads (MiSeq) that was used for polishing of the PacBio data. To obtain the initial assembly of the two hybrid species, *Z. parabailii* and *Z. pseudobailii*, the falcon-based HGAP 4 assembly method in SMRT Link portal v7.0 from Pacific Biosciences was used. As the method already includes the error correction of long reads, polishing was continued with Illumina short reads which has lower base calling error. The assembly was polished three times using Pilon software version 1.22. The inverted full size contigs in the assembly with respect to the reference genome were reverse complemented to make the assembly compatible with public reference. The contigs which were partially inverted were left as they were.

For the assembly of the *Z. bailii* genome, the sequencing library was prepared from PacBio high fidelity (HiFi) reads, with lower error rate (similar to Illumina short reads), hence no polishing was required. HiFi reads were obtained via circular consensus sequencing (CSS) from the SMRT Link portal. The reads were then assembled into the draft assembly using the HiFi option of the Canu

assembler. The draft assembly was polished twice with HiFi reads using Racon software. Then, the repetitive haplotypes were cleaned using the Purge Haplotigs pipeline. The remaining contigs were checked for contamination by aligning the assembly to public reference, and by aligning a part of the contigs to the BLASTn database. Finally, for all final assemblies of the three *Zygosaccharomyces* subspecies, a BUSCO analysis was performed to check the completeness of genomes. The pairwise alignments of genomes were done via the nucmer algorithm from the Mummer 4 package and KeyGene's proprietary STL aligner (multi genome aligner). The multiple genome alignments (as pan genomes) were visualized using Mauve software. All sequences used in this study were submitted to the ENA under the study code PRJEB59101; *de novo* sequences were assigned the accession numbers GCA_949129065, GCA_949129075 and GCA_949129085 for *Z. parabailii* (3699), *Z. bailii* (7846) and *Z. pseudobailii* (3697), respectively.

Genome sequencing of the initial panel of *Zygosaccharomyces* isolates

The 111 isolates from the initial panel (Table S1) were cultured for 24 hours at 25°C in YEPD and genomic DNA (gDNA) was isolated using the CTAB method (Stirling et al. 2003). Some gDNA samples contained evidence of bacterial DNA, therefore, not all strains were included in the library preparation and resequencing. Resequencing was performed by Illumina HiSeq4000 with 2x 125 bp, on two lanes. For all Illumina sequencing, quality control was based on the QC30 threshold (80% of reads with ≤0.1% chance of miscalled base in all lanes of each run). The raw sequence data were trimmed on a minimum base quality of 30 (Phred scores) and sequences with one or more N-nucleotides were discarded. After trimming and filtering, read pairs for which both pairs had a minimum read length of 50 nt were kept for further analysis. The pre-processed sequencing data was mapped to the reference genome using BWA mem 0.7.17. Duplicated reads in the BAM file were marked by Picard-tools MarkDuplicates (v1.63). SAMTOOLS (v1.3) was used to view, index, sort and to merge the alignment files and the BAM files per sample genotyped using GATK 4. Variants such as SNPs and INDELs were identified using GATK4 Haplotypecaller and were stored in a single

Variant Call Format (*.vcf) file. These variants were filtered on allele quality, sample quality and allele depth. A minimum allele quality of 30, minimum sample quality of 20 and minimum allele coverage depth of 7X were used for filtering. SNPs found in all samples that are identical were discarded as they reflect errors in the reference sequence. The filtered variants were annotated using the gene models with SNPeff. Most genes in *Z. parabailii* (3699) and *Z. pseudobailii* (3697) were assigned to the A-subgenome (highly similar to the *Z. bailii* 7846 genome) or to the other subgenome (i.e. B or C; see below). The long sequence aligner Blasr v1.3 was used in the Bidirectional best hit approach to identify homologs. Only coding sequences were used for this approach, extracted from the annotated gene models. Homologous genes with a high sequence similarity (i.e. above 98%) to *Z. baillii* genes were assigned to the A-subgenome and genes below the sequence similarity threshold were assigned to the B-subgenome (hybrid genome of *Z. parabailii*) or C subgenome (hybrid genome of *Z. pseudobailii*). Phylogenetic trees were constructed based on Genetic Distance Analysis of all open reading frames of sequences assigned to the A genome. The A genome of each sequence was compared to the A genome of *Z. parabailii* reference strain 3699 and an Unweighted pair group method with the given arithmetic mean (UPGMA) score (Khan et al., 2008) assigned, from which a phylogenetic tree was constructed (Figure S5). All sequences used in this study were submitted to the ENA under the study code PRJEB59101; strain-specific sample numbers are quoted in Table S1.

Dose-response curve assay for heteroresistance

Yeasts were grown to exponential phase as described above and diluted in YEPD broth to OD_{600} ~0.02 (inoculum size in 75 µl ~10,000 cells) or OD_{600} 0.0002 (inoculum size in 75 µl ~100 cells). The use of the different inocula allowed reliable determination of % viability across a wider range of sorbic acid concentrations. Aliquots (75 µl) of each inoculum size were spread across 90 mm diameter Petri dishes containing 20 ml YEPD agar supplemented with different concentrations of sorbic acid (see above). After 21 days static incubation at 23°C, colony forming units (CFU) were

225 enumerated and percentage survival calculated by reference to control CFU counts on agar without
226 sorbic acid. At least three biological replicates were used in each experiment (exact numbers are
227 clarified in figure legends). Each of the biological replicates (from independent experiments) were
228 the average of three technical replicates for each sorbic acid concentration. Percentage survival data
229 was then fitted to a lognormal distribution curve in R software as detailed below (Fitting to normal
230 and lognormal distributions). Values derived with the curve fitting for IC_{50}, heteroresistance and
231 concentration at which 0.01% survival would be observed (MIC^{MODEL}) were recorded.

232 Inoculum-effect assay for heteroresistance

233 The inoculum effect (Steels et al. 2000) (Box 1) explains how experimentally measured MIC (MIC^{EXP})
234 can increase with size of the cell inoculum, as increased cell number increases the chance that the
235 inoculum includes phenotypically-variant cells that are hyper-resistant. Accordingly, the degree to
236 which the inoculum effect alters MIC^{EXP} is related to the extent of heteroresistance: MIC^{EXP} would
237 not be affected by inoculum size in a completely homogeneous cell population, whereas an impact
238 of increasing inoculum size on MIC^{EXP} will be greater the broader the range of single-cell resistances
239 (Figure 1A). It was reasoned that the magnitude of the inoculum effect could offer a novel,
240 convenient measure of heteroresistance. Dose-response curves of growth-inhibitory effect typically
241 follow a sigmoidal relationship (Stratford et al. 2013b) and have previously been modelled using Hill
242 functions, (Geoghegan et al. 2020, Stratford et al., 2019), indicating a normal frequency distribution
243 of single cell resistances. With that assumption, a relatively small number of MIC^{EXP} determinations
244 at defined inoculum sizes becomes sufficient for curve-fitting (Figure 1B, top panel), to enable
245 determination of standard deviation (σ) from the hypothetical normal distribution of cell resistances
246 (Figure 1B, bottom panel). This standard deviation reflects the extent of heteroresistance, as if the
247 difference between MIC^{EXP} values for different inoculum sizes increases, the normal distribution will
248 have a broader bell curve, reflecting increased heteroresistance. In addition, this curve can be

249 extrapolated to estimate the mean of the normal distribution, reflecting the population-median
250 resistance (IC_{50}, peak of the bell curve).

251 Pre-culture of yeasts was carried out as described above. Exponential phase cells were diluted to
252 OD_{600} ~0.2 (inoculum size in 75µl ~100,000 cells) and serially diluted three times by 10-fold, giving a
253 lowest OD_{600} ~0.0002 (inoculum size in 75µl ~100 cells). Aliquots (75µl) of each inoculum size were
254 transferred to wells of a flat-bottom, 96-well microplate (StarLab) and combined with 75µl of YEPD
255 broth pre-supplemented with sorbic acid at double the desired, final sorbic acid concentration. After
256 sealing with insulating tape, microplates were agitated at 600 rev/min for 60 s, placed in a sealed
257 plastic bag, and incubated statically at 23°C for 21 days. The presence or absence of growth was
258 noted visually under ~2X magnification using a magnifying glass. The lowest concentration at which
259 no growth was detected in ≥1 of four technical replicates was scored as the experimental MIC
260 (MIC^{EXP}) for that inoculum from that biological replicate of the relevant isolate.

261 For inoculum effect measurements with the small panel of *Zygosaccharomyces* strains (Table S3),
262 the procedure was carried out as above but using inoculum sizes of 10^5, 10^4, 10^3 and 10^2 cells/well,
263 with concentration increments of 0.125 mM sorbic acid. For these particular assays, precise
264 inoculum size was verified by plating 75µl aliquots of the OD_{600} ~0.0002 suspension (100 cells) on
265 YEPD agar and incubating at 23°C for 7 days, the corresponding colony count was taken as the
266 inoculum size of the lowest inoculum. The MIC^{EXP} results were fitted to a normal or lognormal
267 distribution curve as described below.

268 Fitting to normal and lognormal distributions

269 Royston et al. (1982) describe a formula to determine the expected value of the maximum of n
270 random variables from a normal distribution ($x_1, .., x_n$), in terms of the theoretical mean (μ) and SD
271 (σ) of that distribution (Eq.1).

$$E[\max(x_1, .., x_n)] = \mu + \sigma * \mathrm{qnorm}((n - \pi/8)/(n - \pi/4 + 1)) \quad \text{(Eq.1)}$$

Assuming that the frequency distribution of antimicrobial resistance of n single cells from a homogeneous cell culture is approximated by a normal (or log-normal) one, this equation can be used to find the IC_{50} (the mean) and heteroresistance (the standard deviation) using data for MIC^{EXP} and their corresponding inoculum sizes (x).

Equation 1 was fitted to experimental data using non-linear least squares fitting, by using the nls() function in R, to return IC_{50} and heteroresistance values for each biological replicate. These were then used to calculate MIC^{MODEL} by taking the concentration value at the 99.99th percentile of the curve (where growth inhibition would occur in 99.99% of cells; Box 1). For each parameter, the mean and standard error of the mean (SEM) across biological replicates were taken.

Plotting of curves for MIC^{EXP} versus inoculum size was carried out using the ggplot package in R. Bootstraps to display 95% confidence interval of the line fitting were calculated using the Boot function in the 'car' package in R.

For dose-response data, sorbic acid concentration values and their corresponding percentage survival values were fitted to one minus a cumulative normal distribution function with mean and standard deviation equal to the IC_{50} and heterogeneity, respectively. Fitting was done using the nls() function in R software. Mean and SEM of each parameter were taken from the fitted curve as for dose response curves detailed above.

Normal or lognormal distribution curves fitted to data were displayed in R (ggplot package), in the case of inoculum effect data, and were displayed using the plot() function in MATLAB R20119b in the case of dose-response curve data. Box and whisker plots, bar charts and correlation plots were produced using Graphpad PRISM 9™ software. Correlations containing points with error bars were produced using Microsoft Excel.

Simulations

Simulation of hypothetical normal distributions for single cell resistances of organisms (for illustration of the inoculum effect concept) were produced using MATLAB R20119b. The Normal Probability Density Function (normpdf) was used to plot frequency distributions for low and high heteroresistance organisms (μ=5, σ=0.1 and μ=5, σ=0.1, respectively) with the frequency distribution divided by ten to simulate the small inoculum size.

Data availability

All data are available in the present results section, in the supplemental material or on request. Genome sequence accession or sample numbers (ENA) are given above and in Table S1.

Results

Use of the inoculum effect for determining heteroresistance

In order to assess the contributions made by phenotypic heterogeneity (specifically, sorbic acid heteroresistance) and IC_{50} to the MIC, across large panels of species or strains, a new assay for measuring these parameters was developed based on the 'inoculum effect' (See methods; Steels et al. 2000), to allow higher throughput than traditional measurements of heteroresistance such as. from agar-based dose-response curve assays (Hewitt et al., 2016). Briefly, this method fitted a normal distribution curve of single cell resistances to experimental MIC (MIC^{EXP}) values, by modelling MIC^{EXP} for a given inoculum as its corresponding point on the normal distribution curve (Figure 1B, top), e.g., MIC^{EXP} for 100 cells/well is the point were 1 in 100 cells would survive, MIC^{EXP} for 1000 cells/well is the point were 1 in 1000 cells would survive, etc. (see Methods). The median and standard deviation of the fitted normal distribution curve (Figure 1B, bottom) could then be taken as the IC_{50} and heteroresistance respectively.

For purposes of curve fitting, lognormal distributions were adopted as these gave better fit to dose response curve data than normal distributions (Figure S1). In addition, possible effect specifically of cell density (cell concentration) on the probability of growth occurring in a well was evaluated, e.g., due to stressor saturation or quorum sensing-type phenomena, as such effect could complicate interpretation of the inoculum effect which infers a total cell number effect. Only a minor possible effect of cell density was apparent, and similarly in high or low heteroresistance isolates (Figure S2). This result supported the inference that differences measured with inoculum effect methodology primarily reflected differences in heteroresistance rather than effects attributable to altered cell density.

To further assess the use of the inoculum effect method, IC_{50} (Figure 2A), heteroresistance (Figure 2B) and MIC (Figure 2C) determinations for six randomly selected strains from the

328 *Zygosaccharomyces* panel (Table 1) were compared between the inoculum effect method and the
329 dose-response curve method. The parameter MIC^{MODEL} was also taken, defined as the lowest
330 concentration giving 99.99% inhibition of cell growth (Box 1) and consistent with previous
331 publications in this field (Stratford et al. 2013b, 2014). This was calculated using the equation for the
332 lognormal distribution curve fitted to MIC^{EXP} data (as in Figure 1B). For inoculum effect assays
333 (carried out in broth), heteroresistance showed a moderately strong, significant correlation with
334 heteroresistance values derived from dose-response curves (r=0.74), while IC_{50} and MIC^{MODEL} also
335 showed strong correlations between the assays (r=0.853 and 0865, respectively). The inoculum
336 effect assay was repeated on agar to assess possible effects of broth- versus agar-based assay
337 formats (as in the above comparison) on the outcomes (Figure S3). Similar correlations were
338 obtained as above indicating that for all the test parameters (IC_{50}, heteroresistance and MIC^{MODEL}),
339 dose response results generally compare well with inoculum effect results obtained either in broth
340 or on agar. These results supported use of the inoculum effect methodology, using a lognormal
341 distribution for data, as an alternative heteroresistance assay to the established, but time-
342 consuming dose-response curve method. Further assays below used the broth condition for
343 inoculum effect assay, which offers higher throughput than on agar and is a condition also more
344 relevant to the beverages and other product types in which sorbic acid is normally used.

345 <u>High-throughput determination of survival parameters for *Zygosaccharomyces* isolates</u>

346 To investigate relationships between heteroresistance or IC_{50} with MIC^{MODEL}, we applied the
347 inoculum effect methodology to a panel of *Zygosaccharomyces* sp. Isolates. To select strains for
348 study, an initial panel of 111 yeasts isolated from foods (see Table S1) were whole-genome
349 sequenced (WGS); most of these had previously been considered as *Z. bailii* isolates (Stratford et al.
350 2013b, 2014, 2019). The sequencing revealed that several isolates previously characterised as *Z.*
351 *bailii* were in fact *Z. parabailii* or *Z. pseudobailii* (see Methods). These species are known interspecies
352 hybrids formed between *Z. bailii* and unknown, closely related ancestors. From these 111 isolates,

353 for further study a panel of 29 strains were selected, to include isolates sampled from across the
354 span of the phylogenetic tree (Figure S5) and diverse sources of isolation. This 'Main
355 *Zygosaccharomyces* panel' comprised 5, 17 and 7 isolates of *Z. bailii*, *Z. parabailii* and *Z. pseudobailii*
356 respectively (Table 1). For parameter measurement (heteroresistance, IC_{50} and MIC^{MODEL}) inoculum
357 effect methodology was used, with curves fitted to MIC^{EXP} values taken at 10^5 and 10^2 cells/well.
358 There was considerable variation in heteroresistance, IC_{50}, and MIC^{MODEL} among isolates of the panel,
359 with ranges of approximately 0.03–0.11, 2.3–5.4 and 4.3–9.2 mM for heteroresistance, IC_{50} and
360 MIC^{MODEL}, respectively (Table 1; Figure 3A). Analysis for each subspecies indicated that *Z. pseudobailii*
361 had a significantly greater mean heteroresistance than *Z. parabailii* and *Z. bailii*, whereas the mean
362 IC_{50} for *Z. pseudobailii* was significantly lower than for *Z. parabailii* (Figure 3B). There were no
363 significant differences in average MIC^{MODEL} between the different subspecies (two sample t-test
364 assuming unequal variances), although there was both a broader spread of values and relatively low
365 mean for *Z. bailii* isolates (Figure 3B).

366 To further investigate the relationships between heteroresistance, IC_{50} and MIC^{MODEL}, the correlation
367 between these parameters was calculated. Across the main *Zygosaccharomyces* panel of 29 strains,
368 strains with high IC_{50} tended to have high MIC^{MODEL} and *vice versa* (r=0.675, p<0.0001), whereas
369 heteroresistance was not significantly correlated with MIC^{MODEL} (r=0.326, p=0.08) (Figure 4A).
370 Unexpectedly, strains with a high IC_{50} tended to have a low heteroresistance (r=0.586, p<0.01)
371 (Figure 4A). When the subspecies were examined independently, *Z. bailii* (Figure 4B), *Z. parabailii*
372 (Figure 4C) and *Z. pseudobailii* Figure 4D) also each showed a significant correlation between IC_{50} and
373 MIC^{MODEL} and no significant correlation between heteroresistance and MIC^{MODEL}. In the case of *Z.*
374 *parabailii*, it was noted that calculated correlations were strongly influenced by a single isolate
375 (3698) with a much lower IC_{50} than all the other *Z. parabailii* isolates (Figure 4C). When the *Z.*
376 *parabailii* dataset was analysed without this single isolate (n=16), heteroresistance was strongly
377 correlated with MIC^{MODEL} (r= 0.660, p<0.01) and IC_{50} was not (r= 0.265, p=0.32) (Figure S4). The
378 correlation between heteroresistance and MIC^{MODEL} in this sample could suggest that

heteroresistance is more strongly related to MIC^{MODEL} when variation in IC_{50} is relatively limited, as was the case in *Z. parabailii* after removal of isolate 3698 (see Discussion).

Trends in heteroresistance, IC_{50} and MIC^{MODEL} across a wide panel of diverse yeast species

To investigate if the trends in sorbic acid heteroresistance described above may be replicated in other spoilage yeast species, dose response curve data from a 'wide panel' of spoilage yeasts isolates (Table S2) produced in a previous survey were analysed. These isolates encompassed 26 species from 12 different yeast genera. Three *Z. parabailii* and two *S. cerevisiae* strains were present in the panel, whereas all other species were represented by one isolate only. To avoid mixing intra- with inter-species comparisons, values averaged across the three *Z. parabailii* and across the two *S. cerevisiae* isolates were used for these two species. This wide yeast panel produced a notably wider range of IC_{50} and MIC^{MODEL} values than the main *Zygosaccharomyces* panel (Figure 5A,B). This was primarily due to comparatively low IC_{50} and MIC^{MODEL} values for certain species of the wide panel, such as *Rhodotorula mucilaginosa* (IC_{50}~0.337mM, MIC^{MODEL}~0.467 mM) and *Zygosaccharomyces rouxii* (IC_{50}~1.34mM, MIC^{MODEL}~2.45mM), two species previously associated with food environments where sorbic acid is not used (Pitt & Hocking, 2009). The two panels exhibited similar variation in heteroresistance between isolates, but the *Zygosaccharomyces* isolates showed significantly higher mean heteroresistance ($p<0.0001$) (Figure 5B). IC_{50} was very strongly correlated with MIC^{MODEL} in the wide panel ($r=0.986$, $p<0.0001$) (Figure 5C). The wide panel also showed a weaker correlation between heteroresistance and MIC^{MODEL} ($r = 0.487$, $p < 0.05$). There was a weak, non-significant correlation between heteroresistance and IC_{50}, which was weaker than that with MIC^{MODEL} suggesting that the relationship between heteroresistance and MIC^{MODEL} was not simply a reflection of the strong relationship between IC_{50} and MIC^{MODEL}. The results collectively indicate a close relationship between MIC and population-median resistance (IC_{50}) and additionally, albeit relatively weakly, with heteroresistance, as discussed below.

404

Discussion

This study showcases the relevance of IC_{50} and heteroresistance parameters for an inhibitor's MIC against organisms, here focussing on the preservative sorbic acid. The novel, high-throughput inoculum effect methodology developed here allowed heteroresistance to be determined in a large number of microorganisms, in a manner less laborious than an established method like dose-response assay on agar. In addition, the microplate format of the new assay demands only modest physical incubator space and is amenable to automation. This methodology offers the potential for screens of heteroresistance in new ecological or industrial contexts, for example in modelling and quantitative risk assessments in the food industry. Our results indicate that a dominant indicator of differences in MIC between strains is differences in IC_{50}, with heteroresistance (e.g. incidence of rare, hyper-resistant cells) playing a smaller role.

The experimental data were consistent with lognormal distributions of single-cell resistances in the yeast cell populations. Hill functions have previously been employed to model dose response curves using $\log_{10}$ values for stressor concentration (Geoghegan et al., 2020; Stratford et al. 2019), a model which assumes single-cell resistances are lognormally distributed. Here, while the fit of the lognormal distribution curve to the data was only marginally better than a normal distribution, for this study we were more interested in differences in relative distributions of single cell resistances, therefore lognormal was suited to the study's aims.

The possibility that cell-concentration may make a contribution to the inoculum effect that is independent of effects due to resistant-cell incidence was investigated, e.g., arising from stressor saturation or quorum sensing-type phenomena, for example. Some previous reports of the inoculum effect have not made this distinction (Loffredo et al. 2020, Smith et al., 2018). However, Scheler et al. (2020) demonstrated that inoculum effect in *E. coli* for cefotaxime resulted from heteroresistance rather than cell concentration, while Steels et al. (2000) ruled out certain effects of cell concentration (e.g. altered adsorption) in the inoculum effect of *Z. bailii* with sorbic acid. On the

other hand, cell concentration affected survival probability of heat-shocked *S. cerevisiae*, attributed to cooperative glutathione excretion at high cell density (Laman Trip & Youk, 2020). To our knowledge, no such mechanisms have been reported for sorbic acid stress. Here, analyses indicated that cell concentration may make only a minor contribution to the increasing sorbic acid MIC^{MODEL} as inoculum size was increased (between 10^2 and 10^4 cells per well). One possible explanation for that small effect could be related to the documented metabolism of sorbic acid by *Z. bailii* (Mollapour and Piper, 2001).

Heteroresistance, IC_{50} and MIC^{MODEL} values obtained on agar (from dose response assay) showed significant correlation with the same parameters obtained in broth (from inoculum effect assay). This consistency for all three parameters whether measured in broth or agar is in line with work elsewhere reporting similar resistances to other stressors in broth versus agar (Morris et al., 2020; Albano et al., 2021; Fuchs et al., 2021). Robustness of the heteroresistance phenotype to the form of growth environment is consistent with heteroresistance being an intrinsic property of cell populations, not strongly affected by environment. This property allows resistant subpopulations to arise independent of stressor exposure, for example, so enabling pre-priming for possible future perturbations. Such pre-priming of heteroresistance has been described for other stressor scenarios (Jing et al. 2018, Levy et al., 2012; Stratford et al. 2014), and is in line with understanding of the wider premise for phenotypic heterogeneity, or bet-hedging (Avery, 2006; Ackermann, 2015).

There was considerable variation in all three parameters between strains and species of the *Zygosaccharomyces* genus and across a wider panel of spoilage yeast species. *Z. bailii* is considered as more heteroresistant to sorbic acid than *S. cerevisiae* (Stratford et al. 2014). Across the main *Zygosaccharomyces* panel and the constituent subspecies, IC_{50} was more clearly correlated with MIC^{MODEL} than was heteroresistance. However, in the case of *Z. parabailii* this trend was reversed by the removal of a single outlying strain with an uncharacteristically low IC_{50} value. The removal of this outlier considerably reduced the variation in IC_{50} for the subspecies (standard deviation reduced to

455 0.055 from 0.076) but had negligible impact on variation in heteroresistance (standard deviations
456 0.0188 and 0.0186). This could suggest that relationships between heteroresistance and MIC are
457 masked when IC_{50} variation is high and potentially dominating effects on MIC across different
458 organisms. Accordingly, heteroresistance may be more important for MIC in species or contexts that
459 support lower variation in IC_{50}.

460 Regarding the question of why one (sub-)species might evolve a higher average preservative
461 heteroresistance than others (e.g. *Z. pseudobailii*, Figure 3B), drivers could be related to the
462 organisms' ecologies or relative incidences. This may include the size of inoculum typically
463 associated with spoilage incidence of an organism, as larger inocula could amplify the effect that
464 heteroresistance has on the MIC. Alternatively, the frequency of transition of the growth
465 environment from preservative-rich to -poor, as frequent changes are thought to favour high
466 heterogeneity (Avery, 2006). Modelling of gene expression patterns has indicated that gene
467 expression noise confers a stronger fitness advantage when population-averaged gene expression is
468 further from the optimal level (Duveau et al. 2018), a situation more often encountered in
469 fluctuating environments. Regarding the particular example of this study, a less stable niche for *Z.*
470 *pseudobailii* is partially supported by this organism being found primarily in viscous foodstuffs (Table
471 1), although a similar argument could be made for *Z. parabailii* (where average heteroresistance was
472 lower) which colonises solid as well as liquid foodstuffs.

473 There was a negative correlation between heteroresistance and IC_{50} across the main
474 *Zygosaccharomyces* panel. One suggestion to explain this might be that heteroresistance is more
475 strongly selected in low-IC_{50} strains in compensation for a disparity between their population-
476 median resistance (IC_{50}) and optimal resistance when faced with stressor. That would be in line with
477 other previously described scenarios, including the example mentioned above where noisy gene
478 expression is especially beneficial when average gene expression is far from the optimum (Duveau et
479 al. 2018).

Other trends were evident here across a wider panel of spoilage yeast species. In particular, a weak but significant positive correlation between heteroresistance and MIC emerged. This suggests that heteroresistance may make a larger contribution to MIC differences between distantly related species of spoilage yeasts than across individual isolates of the *Zygosaccharomyces* genus. At the same time, a correlation between IC_{50} and MIC in the wide panel was stronger than in the main *Zygosaccharomyces* panel, possibly reflecting the wider range of IC_{50} phenotypes across the wide panel. While specific trends were different between the two panels the overall conclusion was similar, that IC_{50} is more closely related than heteroresistance to preservative MIC.

Why heteroresistance has not been strongly associated with sorbic acid resistance in this study requires consideration. One explanation is that if the metabolic cost to a cell of resisting sorbic acid stress is low, the fitness benefit of bet-hedging over increasing population-average resistance would be reduced. While some strategies for sorbic acid resistance such as increased expression of efflux pumps (Geoghegan et al. 2020) would be metabolically costly, favouring heteroresistance (Jing et al. 2018), other proposed resistance mechanisms such as potential maintenance of a more fermentative metabolism (Stratford et al. 2020) might confer resistance at a comparably low cost, mitigating the fitness benefit of heteroresistance over IC_{50}. It is possible that stronger heteroresistance phenotypes would be found in niches where the metabolic cost of pre-priming (all) cells for stress was greater. A possibly-related observation was overrepresentation of the high-IC_{50}, low-heteroresistance species *Z. parabailii* in the initial *Zygosaccharomyces* panel used in this study (n=71, 19 and 21 for *Z. parabailii*, *Z. pseudobailii* and *Z. bailii* respectively, Table S1). As this reflects isolation of *Z. parabailii* from a larger number of food spoilage events, it is consistent with the suggestion that a high IC_{50} as opposed to high heteroresistance may be a more effective evolutionary strategy for growing in preserved foods, though of course we cannot discount that other traits of *Z. parabailii* account for its preponderance. It should also be noted that this study was limited to food spoilage isolates only and other relationships may be manifest in other ecological niches.

Conclusion

We capitalised on the inoculum effect principle to develop a new, high-throughput method for assaying heteroresistance. Application of this assay to diverse spoilage yeasts revealed that heteroresistance and especially IC_{50} are related to MIC, whereas a relationship with heteroresistance was not observed when considering a panel only of *Zygosaccharomyces* sp. isolates. Overall, IC_{50} appears to be a strong indicator of MIC, but heteroresistance may also be related to MIC between distantly related species or in contexts where IC_{50} variation is low. This work highlights certain limitations of conventional MIC measurements as, due to the effect of heteroresistance, the apparent resistance of an organism could vary markedly according to inoculum size. The average resistance of a cell population, its heteroresistance and the inoculum size should all be considered in selecting appropriate antimicrobial concentrations for specific applications, e.g., food preservation. In experimental work (e.g. in microbial challenge testing) this would translate to using an adequate population size (e.g. 10^4 cells) in the desired test volume.

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633 **Table 1: Main panel of *Zygosaccharomyces* strains, including sorbic acid resistance values.**

Strain	Alternative reference	Species*	Isolation Source/location	Heteroresistance (SD, mM)	IC_{50} (mM)	MIC^{MODEL} (mM)
1730		*Z. pseudobailii*	Ketchup	0.0648[a]	3.77	6.69
3697		*Z. pseudobailii*	low fat tartare sauce	0.0899	3.16	7.00
3698		*Z. parabailii*	Chow chow	0.0726	2.41	4.57
3699		*Z. parabailii*	Creamy caesar dressing	0.0453	3.59	5.36
3704		*Z. parabailii*	Ketchup, Russia, 2003	0.0436	4.40	6.47
3942		*Z. parabailii*	Yoghurt mayonnaise	0.0828	3.40	7.07
3959		*Z. parabailii*	Ice Tea concentrate	0.1054	3.56	9.04
7406		*Z. parabailii*	Peach ice tea production environment, Nigeria	0.0688	4.06	7.46
7445		*Z. parabailii*	Ice tea, Mexico	0.0689	3.58	6.58
7768		*Z. bailii*	Spoiled Ice Tea concentrate, Portugal	0.0673	5.06	9.18
7769		*Z. pseudobailii*	Tartare Sauce, Poland, 2019	0.0760	3.63	7.11
7777	DBVPG 6378, CECT11933	*Z. parabailii*	Mayonnaise, Netherlands	0.0456	4.08	6.11
7788	CBS 6708	*Z. bailii*	Orange-juice concentrate, Brazil	0.0502	4.11	6.40
7800	MUCL 38950	*Z. bailii*	fermented tea, 1994	0.0635	2.71	4.76
7807	MUCL 55123	*Z. parabailii*	Alcoholic fermented beverage, 2012	0.0538	3.76	6.05
7809	2, CBS 685, NCYC 563	*Z. pseudobailii*	UK	0.0857	2.32	4.95
7812	6[b,c,d]	*Z. parabailii*	Bottled ice tea, USA	0.0458	4.09	6.13
7820	15[b,c,d]	*Z. pseudobailii*	Salad dressing, Netherlands	0.0719	3.05	5.77
7829	105[b,c,d], NCYC 2933,	*Z. parabailii*	Tomato based product, UK, 2000	0.0897	3.37	7.46
7836	194[b,c,d] DBVPG 6924	*Z. pseudobailii*	Honey, China	0.0643	3.28	5.80

7838	362[b,c,d]	*Z. parabailii*	Factory isolate, Turkey	0.0345	4.35	5.91
7842	592[b,c,d] NCYC 3378	*Z. parabailii*	Soft Drinks factory, Phillipines, 2006	0.0520	4.93	7.82
7843	593[b,c,d] NCYC 3379	*Z. bailii*	Factory isolate, Philippines, 2006	0.0653	2.92	5.20
7851	NCYC 464	*Z. pseudobailii*	Sweet pickle, 1955	0.0935	3.01	6.88
7852	NCYC 573	*Z. bailii*	Red wine, USA, 1959	0.0380	3.06	4.28
7862	NRRL Y-11865	*Z. parabailii*	Salad Cream, 1985	0.0537	3.79	6.09
7870		*Z. parabailii*	Unknown source, Belgium	0.0453	5.38	8.03
7871		*Z. parabailii*	Ice Tea concentrate, Guatemala, 2019	0.0647	4.22	7.49
7873		*Z. parabailii*	Yoghurt, Czech Republic, 2019	0.0637	3.52	6.19

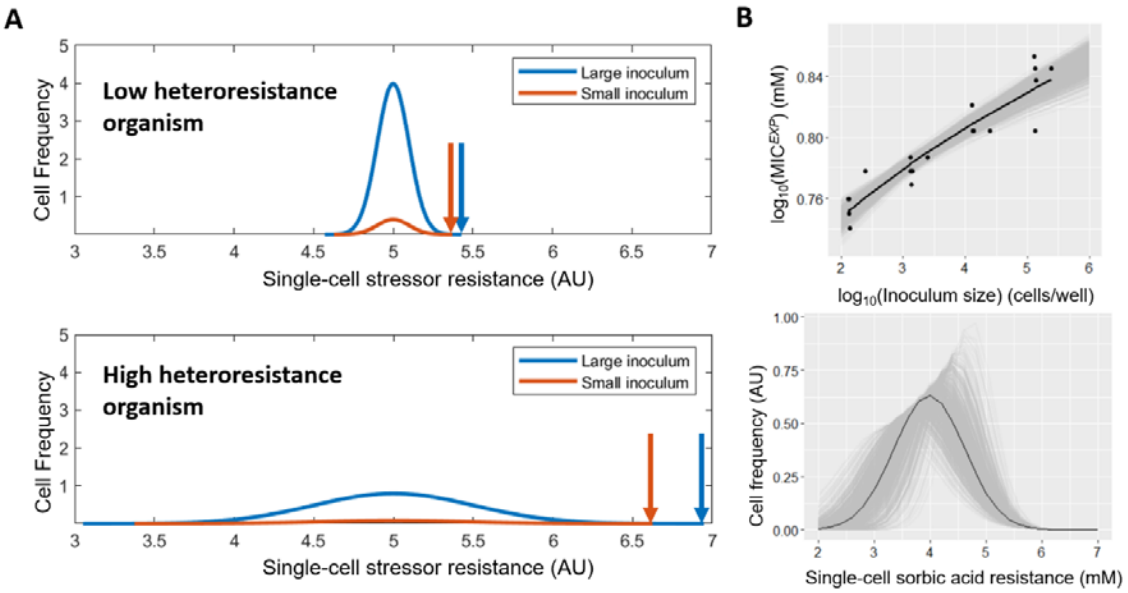
* verified by WGS analysis (see below)

[a] error values for heteroresistance, IC^{50} and MIC^{MODEL} are shown in Figure 3a

[b] as designated by Stratford et al 2019

[c] as designated by Stratford et al. 2020

[d] as designated by Stratford et al 2013b



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642 **Figure 1: Establishing parameters for quantifying heteroresistance *via* the inoculum effect. (A)** Modelled
643 single-cell frequency distributions of stressor resistance (hypothetical) in low- and high-heteroresistance cell
644 populations, at large and small inoculum sizes; these are modelled assuming single-cell resistances are
645 normally distributed in the populations, with the total area under each curve equalling 1 and 0.1 for large and
646 small inocula, respectively. Plots intersect the x-axis where survivor-cell frequency decreases below 0.001,
647 corresponding to the theoretical point at which less than one cell of the relevant inoculum survives and
648 equating to the MIC, indicated with an arrow. AU, arbitrary units. **(B)** Top panel**:** normal distribution curve
649 fitted to MIC^{EXP} (Box 1) values for sorbic acid resistance (mM) at four optical densities (each with six replicates
650 normalised to cells/well according to corresponding colony counts on agar) using inoculum effect methodology
651 (see Methods) for strain 7812 (n=6 biological replicates). Black line, line of best fit. Grey lines, simulated
652 normal distributions, with the distance between the upper and lower lines for each *x* value representing the
653 95% confidence interval for that point. Bottom panel**:** Simulation of frequency distribution of single cell
654 resistances in strain 7812, based on normal distribution curve fitting in panel B (top).

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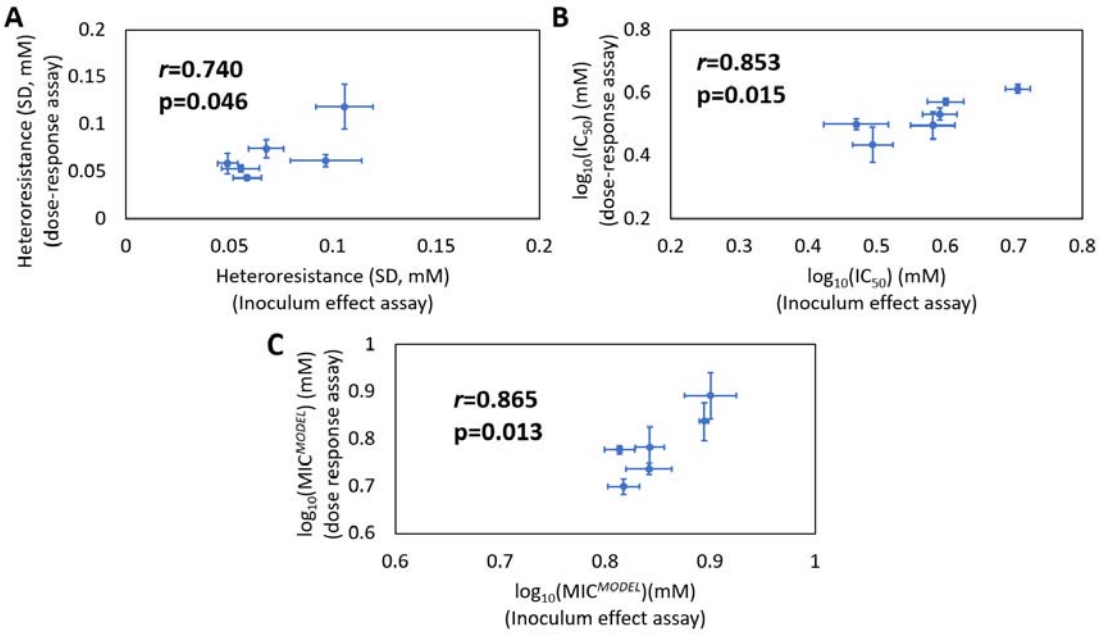

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657 **Figure 2: Corroboration of inoculum effect assay for measurement of IC_{50}, heteroresistance and MIC^{MODEL}.**

658 Comparison of parameter determinations for randomly selected *Z. parabailii*, *Z. bailii* and *Z. pseudobailii*

659 isolates (isolates 7788, 7812, 7851, 7829, 7769, 7842). Values for IC_{50} **(A)**, heteroresistance **(B)** and MIC^{MODEL} **(C)**

660 were derived either from dose-response curve on agar or inoculum effect assay in broth (using MIC^{EXP} values

661 at two inoculum sizes, see Methods). Points represent means from 6 biological replicates for each isolate +/-

662 SEM, with *r* values calculated using linear regression and p values were calculated using one-tailed linear

663 regression.

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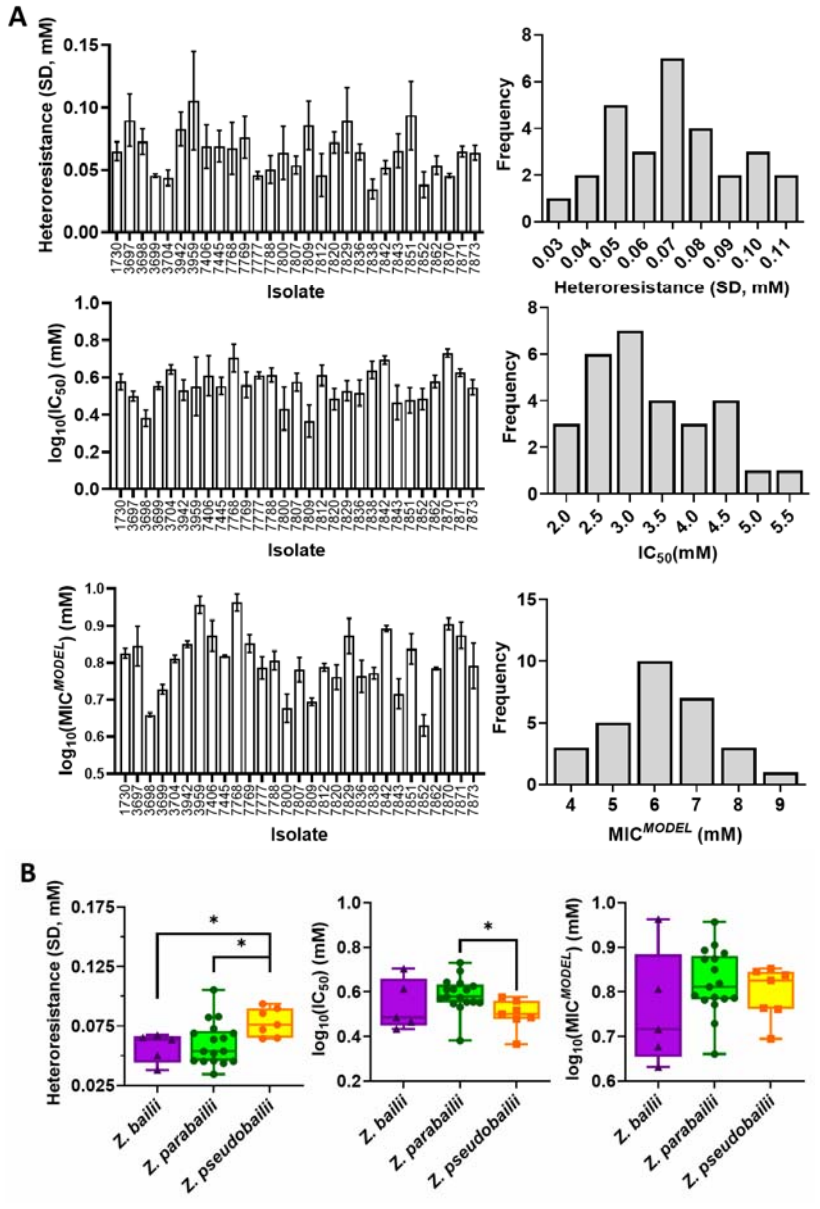

Figure 3: Heteroresistance, IC_{50} and MIC^{MODEL} across a panel of 29 *Zygosaccharomyces* sp. isolates grown with sorbic acid. (A) Left panels: heteroresistance, IC_{50} and MIC^{MODEL} determinations for individual strains. Values are means ± SEM of average of parameters for three curves independently fitted to each biological replicate (MIC^{EXP} values measured at two inoculum sizes.) Right panels: Frequency histograms showing spread of heteroresistance, IC_{50} and MIC^{MODEL} in the 29-strain panel (strain list in Table 1). Values on the x axis indicate centre of each histogram bin, where divisions between bins were at the midpoints between adjacent values shown. **(B)** Data adapted from (A) to compare the distributions of heteroresistance, IC_{50} and MIC^{MODEL} among the isolates of the three subspecies. Significant differences are indicated (*p<0.05), using two-sample *t*-test assuming unequal variances (n =5, 17 and 7, for *Z. bailii, Z. parabailii* and Z. *pseudobailii,* respectively).

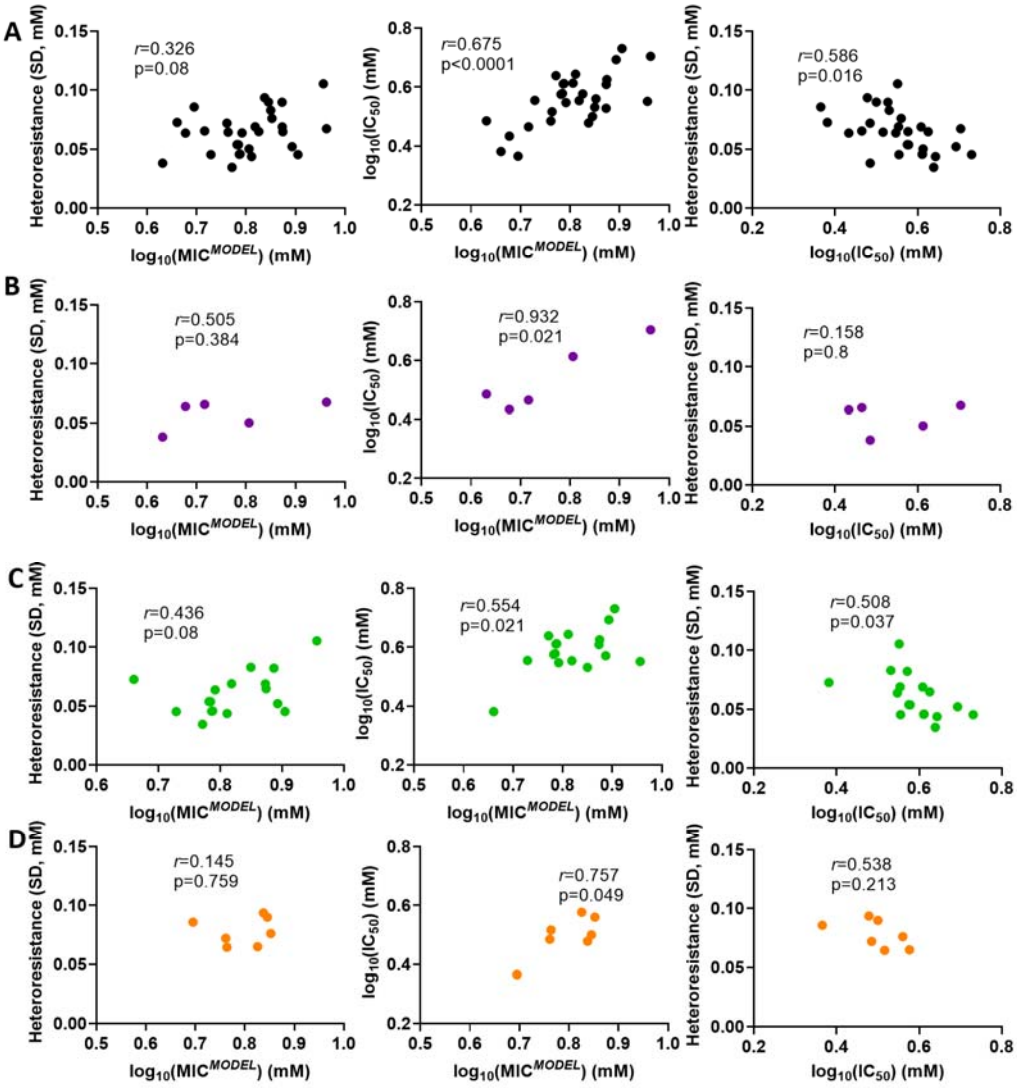

Figure 4: Relationships between heteroresistance, IC_{50} and MIC^{MODEL} in the main panel of *Zygosaccharomyces* sp. isolates. Data were obtained as described in Figure 3. **(A)** Cross-species panel of *Zygosaccharomyces* isolates (n=29) (Table 1). **(B)** *Z. bailii* only (n=5). **(C)** *Z. parabailii* only (n=17). **(D)** *Z. pseudobailii* only (n=7).

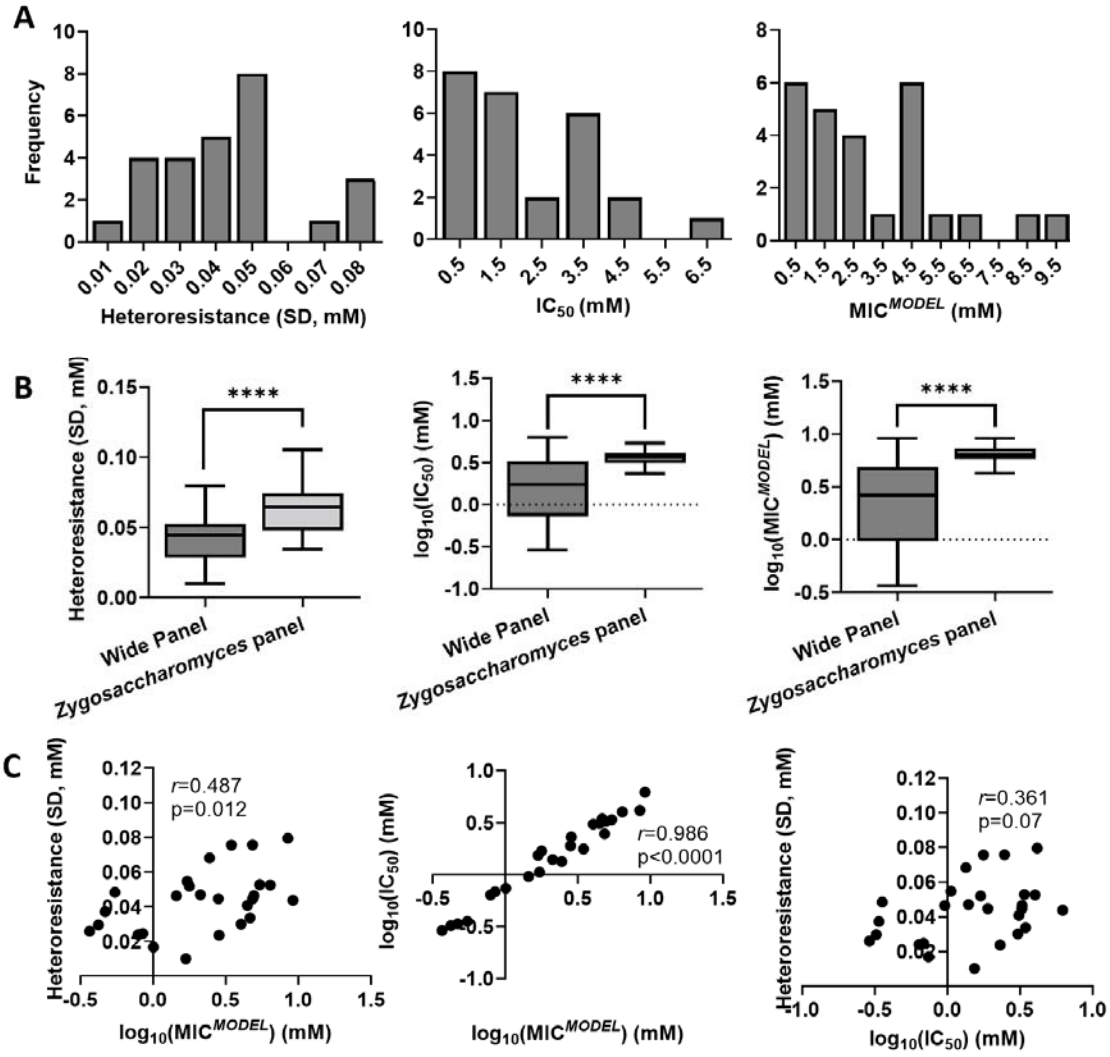

Figure 5: Trends in sorbic acid heteroresistance, IC_{50} and MIC^{MODEL} in a wide panel of diverse yeast species. **(A)** Frequency histograms showing the spread of heteroresistance, IC_{50} and MIC^{MODEL} measured using the dose response curve method. Values for individual 26 species are listed in Table S2. Values for individual isolates were means from three biological replicates. **(B)** Box and whisker plots comparing the parameters between the wide panel (n=26) and the *Zygosaccharomyces* panel (n=29) data from Figure 4. ***, p<0.0001 according to a two-sample *t*-test assuming unequal variances. **(C)** Correlations between heteroresistance, IC_{50} and MIC^{MODEL} for the wide panel.