



Alternative type of Ames test allows for dynamic mutagenicity detection by online monitoring of respiration activity

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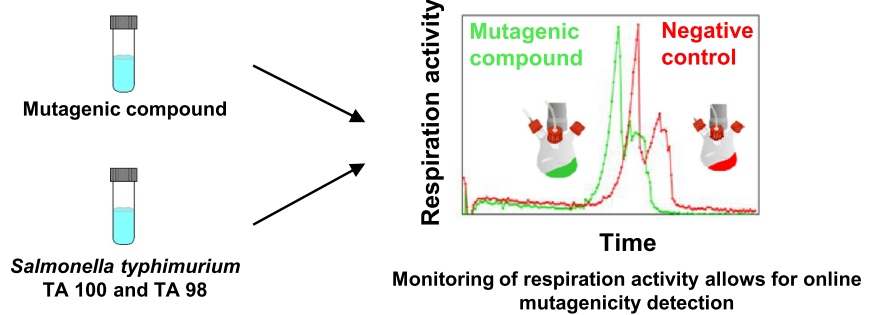
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HIGHLIGHTS

- Online monitoring of respiration activity allows for alternative type of Ames test
- Growth and metabolic activity of *S. typhimurium* were mechanistically modeled
- The model was experimentally validated by monitoring respiration activity
- The acquired data fitted well to results from an Ames fluctuation test

GRAPHICAL ABSTRACT



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ABSTRACT

The Ames test is the most commonly used mutagenicity test worldwide. It is based on a microbial system that uses histidine auxotrophic *Salmonella typhimurium* strains. Due to either spontaneous mutations or mutations induced by a mutagenic compound, the cells can regain their ability to grow without histidine supplementation. The degree of mutagenicity of a sample correlates with the number of cells that are able to grow in media that lack histidine. All test variants published up to now are endpoint determinations providing no information about cell growth and respiration activity during the cultivation time. This study aimed to develop an alternative type of Ames test by characterizing the respiration activity of *Salmonella typhimurium* over time for dynamic mutagenicity detection. It focuses on elucidating the mechanisms underlying this novel test system, and serves as a general proof of principle.

Respiration activity (oxygen transfer and uptake rate) and biomass growth of *Salmonella typhimurium* TA 100 and TA 98 were mechanistically modeled to understand and predict the behavior of the bacteria during the Ames test. The results simulated by the model were experimentally validated by the online monitoring of respiration activity over cultivation time using a Respiration Activity Monitoring System (RAMOS). The simulated prediction was observed to fit well to the experimental data. When a mutagenic compound was added, its mutagenicity could be detected online due to the elevated cell number and respiration of histidine prototrophic cells. Laborious manual

Abbreviations: DMSO, dimethyl sulfoxide; FAU, formazine attenuation units; OD, optical density; OTR, oxygen transfer rate; OUR, oxygen uptake rate; RAMOS, Respiration Activity Monitoring System.

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evaluation of mutagenicity after cultivation is not necessary. Mutagenicity evaluation with the presented alternative Ames RAMOS test fitted well to results from an Ames fluctuation test. In the future, a miniaturized RAMOS device for microtiter plates should allow for a high-throughput Ames RAMOS test.

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1. Introduction

The Ames test, also known as bacterial reverse mutation assay (MacGregor et al., 2000), Salmonella mutagenicity assay (Claxton et al., 2010) or reversion assay, is used worldwide and is a widely accepted mutagenicity test (Mortelmans and Zeiger, 2000; Mortelmans, 2019; Zeiger, 2019). It detects point mutations in genes involved in histidine biosynthesis. Although the use of quantitative structure-activity relationship (QSAR) models is of increasing importance, the Ames test is an essential initial screening tool used to test new chemicals such as food contact materials and drugs for mutagenicity, and is also used to evaluate environmental samples (Mortelmans and Zeiger, 2000; Umbuzeiro et al., 2016; Rainer et al., 2018; Van Bossuyt et al., 2018; Honma et al., 2019; Rainer et al., 2019). Claxton et al. (2010) stated that the Ames test is the “stethoscope of the 21st century” and counted >10,000 publications about the Ames test until 2010. Due to the emerging field of green toxicology (Crawford et al., 2017), as well as the revision of the water framework directive (Brack et al., 2018; Brack et al., 2019), more publications on the Ames test are expected in the future. The Ames test is also widely used in industry, pointing to the existence of additional partially unpublished data. Advantages of the Ames test include its simplicity, cost effectiveness, flexibility, and its large validated database compared to other mutagenicity tests (Claxton et al., 2010; Levy et al., 2019). This test is based on a microbial system that uses histidine auxotrophic *Salmonella typhimurium* strains. Due to their acquired DNA repair deficiency, they are especially sensitive to mutations (Ames et al., 1973; Ames et al., 1975; McCann et al., 1975; Maron and Ames, 1983), with the exception of the strain TA 102 (Mortelmans and Zeiger, 2000). Cells which mutate back to histidine prototrophy, can grow without histidine. The mutation in the histidine operon differs between the used test strains (Ames et al., 1973; McCann et al., 1975; OECD, 1997).

The standard Ames test is carried out on agar plates (Ames et al., 1973). The plate incorporation version of the Ames agar plate test has been standardized by the OECD in 1997 in terms of medium composition, test strains used, and cultivation conditions, as shown in Fig. 1.

Performing the Ames test on agar plates is highly labor-intensive, though it is less labor-intensive than *in vivo* mutagenicity tests. As an alternative to the Ames test on agar plates, the Ames fluctuation test was developed. It is less laborious and is performed in liquid cultures in microtiter plates. It was firstly described by Green et al. (1977a, 1977b) and has been repeatedly used (Gatehouse, 1978; Pérez et al., 2003; Reifferscheid et al., 2005; Reifferscheid et al., 2011; Proudlock and Evans, 2016) and validated (Umbuzeiro et al., 2010; Flückiger-Isler and Kamber, 2012; Reifferscheid et al., 2012). It was standardized in 2012 by ISO 11350, as shown in Fig. 2 (ISO, 2012). A pH decrease detected by a pH indicator is indicative of growth. Instead of the five strains used in the plate incorporation assay, only two strains are recommended for this test (McCann et al., 1975; ISO, 2012), namely *S. typhimurium* TA 100 (with a base-pair substitution in histidine operon) and *S. typhimurium* TA 98 (with a frameshift in histidine operon). Recent publications by the U.S. EPA indicate that this reduced set of strains is sufficient to conduct a sound mutagenicity assessment (Williams et al., 2019).

Even though the Ames fluctuation test is less laborious and leads to less material consumption than the Ames test on agar plates, it still requires a large quantity of both disposables and time. Improved alternative test methods are, therefore, required. This was also noted by Zwart et al. (2018) and led to the development of a further downscaled and

shorter Ames fluctuation test: Luminescent derivatives of the test strains TA 100 and TA 98 were constructed. Luminescence was correlated to revertant growth. Although luminescence was monitored online during test development, the luminescent Ames test is also an endpoint determination, measuring luminescence only once after 28 h. The more luminescence is measured at this endpoint, the more growth of revertants is assumed (Zwart et al., 2018). Thus, the currently published Ames test variants are endpoint determinations that provide no information about the growth kinetics and metabolic activity of the bacteria during testing. In addition, for any type of Ames fluctuation test, a transfer of each culture between the exposition and incubation phase is necessary to spread the cultures into several wells, as can be seen in Fig. 2.

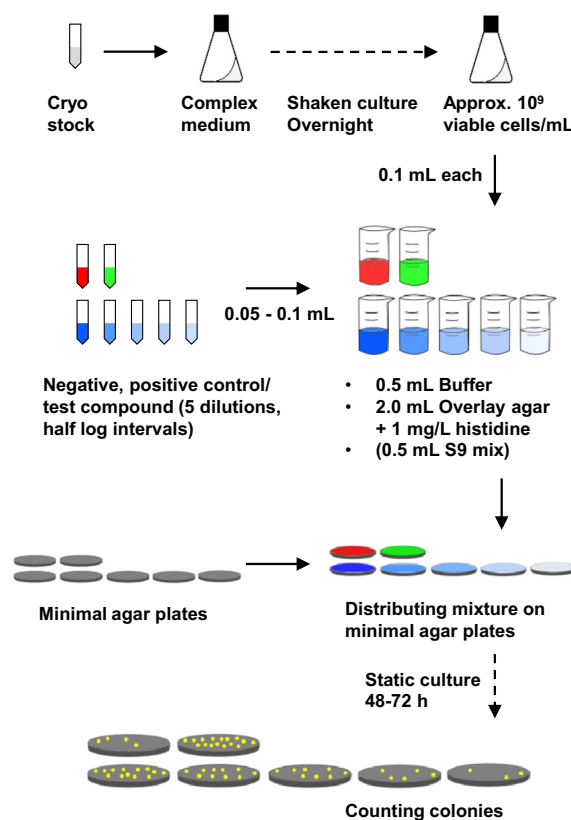


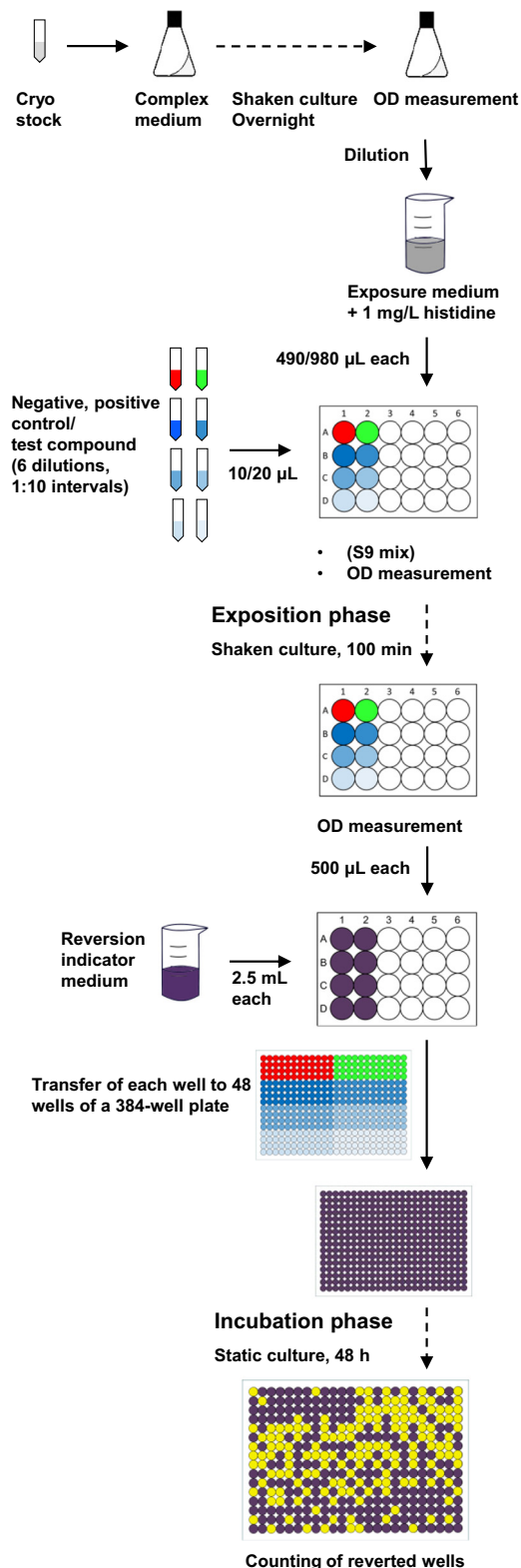
Fig. 1. Graphical illustration of the standard Ames test on agar plates, as defined in OECD guideline 471 (OECD, 1997) (plate incorporation test). Complex medium is inoculated with a cryo stock of either *S. typhimurium* TA 1535, TA 1537, TA 100, TA 98 or TA 102. After overnight growth (termination in late exponential or early stationary phase; shaking parameters not defined in OECD guideline 471 (OECD, 1997)), the cells are mixed with negative control (red)/positive control (green)/test compound in five dilutions (blue), buffer and overlay agar containing 1 mg/L histidine. After plating on minimal agar plates (volume not defined in OECD guideline 471 (OECD, 1997)), the histidine auxotrophic cells can grow until histidine is depleted. Only reverted cells can continue to grow and form colonies. The number of colonies correlates with the mutagenicity of a test compound. Each strain has to be tested without and with S9 mix (metabolic activation system). This leads to a minimum total number of 84 agar plates/test compound for only two strains tested in triplicate. For all five strains, 210 agar plates are necessary. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The growth kinetics of *S. typhimurium* during the Ames test can be mathematically described. So far, the Ames test has been mainly modeled on a statistical and not on a kinetic level (Margolin et al., 1981; Salmeen and Durisin, 1981; Krewski et al., 1993); however, in this study, the Ames test was modeled on a kinetic level. Growth and

respiration activity over time were described for both negative (no mutagenic compound) and positive (mutagenic compound) controls.

Kinetic information can be obtained experimentally by online monitoring of the respiration activity over cultivation time. Respiration activity is quantified through the oxygen transfer rate (OTR), which can be measured with a Respiration Activity Monitoring System (RAMOS) in shake flasks (Anderlei and Büchs, 2001; Anderlei et al., 2004; Lattermann and Büchs, 2016). The OTR correlates to the respiration activity of aerobic cells and gives insight into the metabolism and growth of microorganisms (Anderlei and Büchs, 2001; Anderlei et al., 2004). Exponential biomass growth correlates with an exponential OTR increase. Thus, the OTR is a measurand for bacterial growth. In the field of biochemical engineering, the investigation of the OTR over cultivation time is often used to optimize bioprocesses in terms of medium composition and productivity. It can also be used to monitor the OTR of histidine prototrophic bacteria on a medium lacking histidine to evaluate the number of evolved revertants in the Ames test. The more revertants are present after histidine depletion, the earlier the biomass concentration and the OTR of the histidine prototrophic cells visibly increase.

Therefore, the aim of this research was to present the fundamental concept of an alternative type of Ames test (Ames RAMOS test), which involves online monitoring the OTR of *S. typhimurium* strains TA 100 and TA 98 over time for dynamic mutagenicity detection without an additional incubation phase. Modeled data for a shake flask cultivation was validated against the experimental data obtained using a RAMOS device. The results were then compared to results from an Ames fluctuation test. The generation of dose response data, as well as the investigation of reproducibility, is published subsequently.



2. Material & methods

2.1. Microorganisms and cultivation conditions

Commercially available Ames test strains *S. typhimurium* TA 100 and TA 98 were purchased from Trinova Biochem GmbH (Giessen, Germany) and were used in this study. The strains are described in detail in ISO 11350 (2012) and by Reifferscheid et al. (2012). Preculture was carried out in accordance with ISO 11350 (2012) and Reifferscheid et al. (2012). About twenty (20) mL of growth medium as described in Section 2.2 was inoculated with 20 µL of cryopreserved stock, and was cultivated at 37 °C. Instead of using 100 mL Erlenmeyer flasks as is specified in ISO 11350 (2012), 250 mL RAMOS flasks were used to monitor the OTR online. Instead of shaking at 150 rpm as specified in ISO 11350 (2012), 250 rpm with a shaking diameter of 50 mm was used, which assured oxygen unlimited conditions. The cells were cultivated for 7–8 h, depending on when the second exponential growth ended (see supplementary file 1). ISO 11350 (2012) only requires the incubation of not more than 10 h for preculture cultivation; thus, incubation time can vary between laboratories. Long cultivation

Fig. 2. Graphical illustration of the Ames fluctuation test in microtiter plates, as defined in ISO guideline 11350 (ISO, 2012) and described by Reifferscheid et al. (2012). Complex medium is inoculated with a cryo stock of either *S. typhimurium* TA 100 or TA 98. After overnight growth (termination after max. 10–12 h; shaking parameters vaguely defined in ISO 11350 (ISO, 2012)), the cells are diluted to 45 FAU (TA100)/180 FAU (TA 98) with exposure medium containing 1 mg/L histidine and filled into a 24-well plate. Negative control (red)/positive control (green)/test compound in six dilutions (blue) are added. The initial optical density is measured, if a cytotoxicity check is done (1 mL/well instead of 0.5 mL/well is needed for cytotoxicity testing). The cells can grow until histidine is depleted. After 100 min the optical density is measured again to detect cytotoxic effects. Afterwards reversion indicator medium containing the pH indicator bromocresol purple is added and each culture is transferred to 48 wells of a 384-well plate. Only reverted cells can continue to grow and shift the pH to lower values, which leads to a color change from purple to yellow. The number of color-changed wells correlates with the mutagenicity of a test compound. Each strain has to be tested without and with S9 mix. This leads to a minimum total number of four 24-well plates and twelve 386-well plates/test compound for triplicate testing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

times of up to 10 h are most likely recommended due to oxygen limited conditions resulting from the specifications of the Ames fluctuation test. Oxygen limitation leads to reduced growth rates.

An alternative type of Ames test (Ames RAMOS test) with online monitoring of respiration activity was developed as shown in Fig. 3. Instead of a separate exposition and incubation phase as in the Ames fluctuation test (ISO, 2012; Reifferscheid et al., 2012), the cells were cultivated in an extended test culture without further dilution in a medium with a pH indicator. The preculture biomass was diluted to 45 ± 5 formazine attenuation units (FAU) for *S. typhimurium* TA 100 and 180 ± 10 FAU for *S. typhimurium* TA 98. This was done according to Reifferscheid et al. (2012), because strain TA 100 has a higher spontaneous mutation rate than TA 98 (McCann et al., 1975). Since both strains

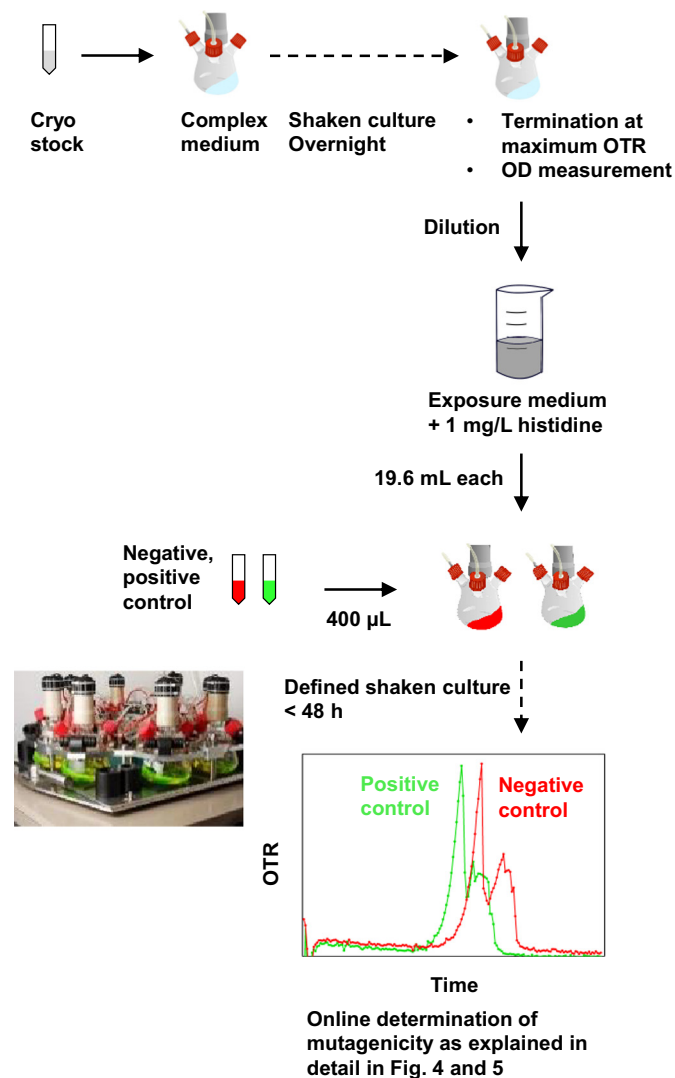


Fig. 3. Graphical illustration of the newly developed Ames RAMOS test. It allows the online investigation of growth and respiration of *S. typhimurium* test strains. For proof of concept, only a negative and a positive control are investigated. Complex medium is inoculated with a cryo stock of either *S. typhimurium* TA 100 or TA 98. After overnight growth under defined shaking and termination conditions (see supplementary file 2) in a RAMOS device, the cells are diluted to 45 FAU (TA 100)/180 FAU (TA 98) with exposure medium (minimal medium) containing 1 mg/L histidine and are transferred into RAMOS flasks. Negative control (red)/positive control (green) are added, followed by shaken cultivation for 45 h (test culture). The oxygen transfer rate (OTR) in the RAMOS device is monitored to detect mutagenicity. The time difference in the increase of the OTR correlates with the mutagenicity of a test compound. The earlier the OTR increases in contrast to the negative control, the more mutagenic is a compound. Shaking conditions: 250 rpm, 50 mm shaking diameter, filling volume 20 mL (250 mL flasks), 37 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

have similar final cell densities in the preculture, the strains have to be diluted in different ratios in order to achieve the mentioned initial cell densities in the test. For the test culture with strain TA 100, $1 \times$ concentrated minimal medium was used, while for strain TA 98, $1.2 \times$ concentrated minimal medium was used. A positive control compound and a negative control compound were added to the cultivation medium in the same volume ratio of 1:50 as in the Ames fluctuation test (ISO, 2012). For each of the experimental conditions, duplicates of the test culture were cultivated in a RAMOS device with parallel cultivations in normal Erlenmeyer flasks for offline sampling. After each offline sampling, the corresponding flask was discarded (Wewetzer et al., 2015). To ensure equal conditions in the offline and online cultivations, a master mix with preculture, minimal medium and control compound was prepared, which was then distributed to the different flasks. All flasks had a total volume of 250 mL and were filled with 20 mL of cultivation broth. They were cultivated at 37 °C with 250 rpm and a shaking diameter of 50 mm. Cultivations were stopped after 45 h.

2.2. Media composition and control compounds

All chemicals were obtained from Sigma Aldrich (Darmstadt, Germany) with a purity of at least 97.5%, unless otherwise stated.

Media for the Ames RAMOS test were developed based on the Ames fluctuation test, which is also conducted in liquid culture. For preculture cultivation, the growth medium was prepared as described in ISO 11350 (2012) and Reifferscheid et al. (2012). This medium consisted of 18.8 g/L Oxoid nutrient broth No. 2 (Thermo Fisher Scientific, Waltham, USA) and 1.24 g/L NaCl dissolved in deionized water. The pH value was adjusted to 7.5. Notably, for Ames agar plate test precultures, NaCl is not additionally supplemented. For the test culture, a minimal medium was used. It contained 4.3 g/L D-glucose, 2.2 g/L citric acid-monohydrate, 10.8 g/L K_2HPO_4 , 3.8 g/L $NaNH_4HPO_4 \cdot 4 H_2O$, 0.2 g/L $MgSO_4 \cdot 7 H_2O$, 0.0026 g/L D-biotin, and 0.001 g/L L-histidine. Chemicals correspond to the exposure medium used in the Ames fluctuation test. Concentrations used correspond to the reversion indicator medium used in the Ames fluctuation test (ISO, 2012), with the exception of histidine. The pH value was adjusted to 7.0 using NaOH. All chemicals used were dissolved in deionized water.

As negative control, pure DMSO was used, as the positive control compound is dissolved in this solvent. For the general proof of principle for the Ames RAMOS test, only one standard concentration of a positive control per strain was used. For test strain TA 100, a stock of 12.5 mg/L nitrofurantoin dissolved in DMSO was used as a positive control, which resulted in a test concentration of 0.25 mg/L. For test strain TA 98, a stock of 0.5 g/L 4-nitro-*o*-phenylenediamine dissolved in DMSO was used as a positive control, which resulted in a test concentration of 0.01 g/L. Both controls comply with ISO 11350 (2012). As such, Ames RAMOS test results and Ames fluctuation test results are more easily compared.

2.3. Oxygen transfer rate measurement in a RAMOS device

Both, the preculture and test culture were monitored online in an in-house developed RAMOS device that allows online monitoring of OTR in shake flasks (Anderlei and Büchs, 2001; Anderlei et al., 2004). Eight shake flasks can be monitored in parallel. The RAMOS device is commercially available from Kühner AG (Birsfelden, Switzerland) or HiTec Zang GmbH (Herzogenrath, Germany).

2.4. Offline analysis

Parallel to online measurement, offline samples from separate Erlenmeyer flasks were taken at characteristic time points of the OTR curve. By taking offline samples, conclusions drawn from online measurements can be checked. In addition, further parameters like pH value can be evaluated, leading to a deeper understanding on the metabolic

activities taking place during cultivation. Optical density (OD) at 595 nm was determined using standard 1 mL cuvettes (Genesys 20 photometer, Thermo Scientific, Germany). If an OD value was above 0.35, the culture broth was appropriately diluted with 0.9% NaCl to keep the measured OD within the linear range of the photometer. Obtained OD values were converted to FAU values. Biomass (cell dry weight) was determined by centrifuging 2 mL of culture broth in a dry reaction tube (14,000 rpm, 10 min), after which the supernatant was discarded, and the pellet was dried. Biomass was determined by the mass difference of the reaction tube before and after the procedure. If the morphology of the bacteria remains constant during cultivation (which is the case for *S. typhimurium* during the Ames test), the optical density (measured in FAU) should be proportional to the biomass, (measured in g/L). The pH was measured with a CyberScan pH 510 device (Eutech Instruments, The Netherlands).

3. Calculation

To model the respiration activity (OTR) and growth of *S. typhimurium* TA 100 and TA 98 in the test culture of the Ames RAMOS test, a co-cultivation model of histidine auxotrophic and histidine prototrophic cells was used. Histidine auxotrophic cells represent the purchased Ames test strains, while histidine prototrophic cells represent the back mutated revertants, which appear due to spontaneous or induced mutation. It is assumed that cells can only mutate while they are replicating (growing), and that no histidine prototrophic cells are present at the beginning of the cultivation. However, this is a simplification since there are always some histidine prototrophic bacteria (revertants) present in the inoculum. Since realistic numbers of histidine prototrophic bacteria did not show any significant effects to the results of the model calculation (data not shown), they were neglected here.

With these assumptions, the derivative of histidine auxotrophic biomass concentration over time (dX_{His}/dt) can be explained by Eq. (1).

$$dX_{His}/dt = \begin{cases} -k_d \cdot X_{His} & \text{if } \mu_{His} = 0 \\ \mu_{His} \cdot X_{His} - k_d \cdot X_{His} - m \cdot X_{His} & \text{if } \mu_{His} > 0 \end{cases} \quad (1)$$

As long as the growth rate μ_{His} of histidine auxotrophic cells equals zero, dX_{His}/dt is only determined by the death rate k_d , which describes the rate at which cells lyse. It is reasonable to assume that there is always a specific portion of lysing cells in a bacterial culture. If μ_{His} is larger than zero, dX_{His}/dt is determined by k_d , μ_{His} , and the mutation rate m . At this rate, histidine auxotrophic cells mutate back to histidine prototrophy. The mutation rate m is dependent on the spontaneous mutation rate and on the mutagenic potency and concentration of the added sample. If μ_{His} equals zero, the overall mutation rate also equals zero since replication (= growth) has to occur in order to manifest a mutation. This results in Eq. (2), which shows the derivative of histidine prototrophic biomass concentration over time (dX/dt).

$$dX/dt = \begin{cases} \mu \cdot X - k_d \cdot X & \text{if } \mu_{His} = 0 \\ \mu \cdot X - k_d \cdot X + m \cdot X_{His} & \text{if } \mu_{His} > 0 \end{cases} \quad (2)$$

If μ_{His} is larger than zero, dX/dt is not only dependent on k_d and the growth rate μ for histidine prototrophic cells, but also on the mutation rate m at which histidine auxotrophic cells become histidine prototrophic. The growth rates of the two types of cells are defined in Eqs. (3) and (4), following Monod kinetics (Villadsen et al., 2011).

$$\mu_{His} = \mu_{max} \cdot Glc / (Glc + K_{Glc}) \cdot His / (His + K_{His}) \quad (3)$$

$$\mu = \mu_{max} \cdot Glc / (Glc + K_{Glc}) \quad (4)$$

μ_{His} is dependent on the carbon source (glucose) and histidine concentration, since it describes the growth of histidine auxotrophic cells. As soon as histidine is depleted, the growth rate of the histidine

auxotrophic cells equals to zero. μ is only dependent on glucose concentration, since it describes the growth of histidine prototrophic cells that grow in the absence of histidine, as they are not dependent on the histidine in the medium.

Eq. (5) shows the derivative of glucose concentration over time ($dGlc/dt$). Glucose is consumed due to growth and maintenance (m_{Glc}). Thus, the standardized initial concentration of glucose decreases depending on the metabolic activity of the bacteria. $Y_{X/Glc}$ is the yield coefficient of biomass on glucose.

$$dGlc/dt = -1/Y_{X/Glc} \cdot \mu_{His} \cdot X_{His} - m_{Glc} \cdot X_{His} - 1/Y_{X/Glc} \cdot \mu \cdot X - m_{Glc} \cdot X \quad (5)$$

Eq. (6) shows the derivative of histidine concentration over time ($dHis/dt$). It has to be noted that a small amount of histidine (1 mg/L) is present at the beginning of the culture in order to allow a few cell divisions of the histidine auxotrophic bacteria. Otherwise, no mutations can be fixed. It is assumed that only histidine auxotrophic cells consume histidine. Consequently, the standardized initial concentration of histidine decreases dependent on the metabolic activity of the histidine auxotrophic bacteria. $Y_{X/His}$ is the yield coefficient of biomass on histidine.

$$dHis/dt = -1/Y_{X/His} \cdot \mu_{His} \cdot X_{His} \quad (6)$$

To model the breathing activity of the cells apart from the biomass, the dissolved oxygen concentration in the liquid phase (O_2), oxygen transfer rate (OTR), and oxygen uptake rate (OUR) are calculated. Eq. (7) describes the derivative of the dissolved oxygen concentration over time (dO_2/dt).

$$dO_2/dt = OTR - OUR_{His} - OUR \quad (7)$$

OTR describes the total transfer rate of oxygen from gas to liquid phase (Anderlei and Büchs, 2001; Villadsen et al., 2011). The oxygen uptake rate from the liquid phase by the cells is denoted as OUR. OUR is separately modeled for both types of cells. OUR_{His} describes the oxygen uptake rate of histidine auxotrophic cells, while OUR describes it of histidine prototrophic cells. Since the absolute change in dissolved oxygen concentration is negligibly small in comparison to OTR and OUR (Mühlmann et al., 2018), it can be assumed that OTR describes the overall oxygen consumption of the auxotrophic and prototrophic cells (overall oxygen uptake rate) and, therefore, the respiration activity of all aerobic cells in the liquid.

OTR can generally be described by Eq. (8) and is determined by the volumetric mass transfer coefficient $k_L a$ and the gradient of the molar fraction of oxygen between the gas and liquid phase (Maier and Büchs, 2001; Lattermann and Büchs, 2016).

$$OTR = k_L a \cdot L_{O_2} \cdot p \cdot (y_{out} - y) \quad (8)$$

The volumetric mass transfer coefficient $k_L a$ describes how efficiently oxygen is transported from the gas to the liquid phase. k_L is the mass transfer coefficient and a is the specific surface area between the gas and liquid phase (transfer area/liquid volume) (Villadsen et al., 2011). L_{O_2} is the solubility of oxygen in the aqueous liquid, p is the absolute pressure, y describes the molar fraction of oxygen in the liquid, and y_{out} describes the molar fraction of oxygen in the head-space of the shake flask, which is dependent on OTR. Eq. (9) can be used to calculate y_{out} .

$$y_{out} = y_{in} - OTR \cdot V_{mo} / q_g \quad (9)$$

y_{in} describes the molar fraction of oxygen in the supplied gas, q_g represents the aeration rate of the RAMOS device, and V_{mo} represents the molar gas volume. By inserting Eq. (9) into Eq. (8) and rearranging the

terms, Eq. (10) is derived for the calculation of OTR.

$$OTR = q_g \cdot k_L a \cdot L_{O_2} \cdot p \cdot (y_{in} - y) / (q_g + V_{mo} \cdot k_L a \cdot L_{O_2} \cdot p) \quad (10)$$

OUR_{His} and OUR are calculated according to Eqs. (11) and (12), where M_{Glc} is the molecular weight of glucose.

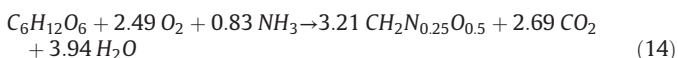
$$OUR_{His} = 1/Y_{X/Glc} \cdot \mu_{His} \cdot X_{His} \cdot 1/M_{Glc} \cdot v_{O_2, growth} + m_{Glc} \cdot X_{His} \cdot 1/M_{Glc} \cdot v_{O_2} \quad (11)$$

$$OUR = 1/Y_{X/Glc} \cdot \mu \cdot X \cdot 1/M_{Glc} \cdot v_{O_2, growth} + m_{Glc} \cdot X \cdot 1/M_{Glc} \cdot v_{O_2} \quad (12)$$

Oxygen uptake and consumption are dependent on growth and maintenance of the cells. The stoichiometric coefficients for only respiration (v_{O_2}) and respiration and growth ($v_{O_2, growth}$) differ. Eq. (13) shows the stoichiometric equation for the pure respiration on glucose with a stoichiometric coefficient for oxygen v_{O_2} of 6.



Assuming an average bacterial biomass composition (Shaw et al., 2009), a stoichiometric coefficient for oxygen ($v_{O_2, growth}$) of 2.49 was calculated as shown in Eq. (14). NH_3 is the nitrogen source (added as medium ingredient $NaNH_4HPO_4$). For simplification, only glucose is assumed as a carbon source. Citrate, which is present in the medium in a significant amount, is only consumed after glucose is depleted. This later phase of the culture is irrelevant for the Ames RAMOS test and is therefore, neglected.



All variables used to model the respiration activity and growth of *S. typhimurium* are summarized in Table 1.

Table 2 shows the constant values used. Values were either experimentally acquired by conducting growth experiments in a RAMOS device (data not shown) and fitted to the previously acquired growth curves or were derived from literature. The initial medium concentrations were taken from the used cultivation medium with standard initial glucose and histidine concentrations, which was derived from the Ames fluctuation test medium composition.

Used test strains differ in initial concentration, as described in Section 2.1. To model the Ames RAMOS test using both strains, strain-specific initial concentrations of histidine auxotrophic cells were used. For strain TA 98, an initial concentration of 0.21 g/L was used and for strain TA 100, an initial concentration of 0.06 g/L was used. Simulation of growth and respiration of the Ames test strains was performed with ModelMaker 3.0.4 (Cherwell Scientific Ltd., The United Kingdom).

Table 1
Variables for modeling the Ames RAMOS test.

Constant	Abbreviation	Unit
Histidine auxotrophic cell concentration	X_{His}	g/L
Histidine prototrophic cell concentration	X	g/L
Growth rate of histidine auxotrophic cells	μ_{His}	1/h
Growth rate of histidine prototrophic cells	μ	1/h
Glucose concentration	Glc	g/L
Histidine concentration	His	g/L
Oxygen concentration in the liquid	O_2	mol/L
Oxygen transfer rate	OTR	mmol/L/h
Oxygen uptake rate of histidine auxotrophic cells	OUR_{His}	mmol/L/h
Oxygen uptake rate of histidine prototrophic cells	OUR	mmol/L/h
Molar fraction of oxygen in the head-space of the shake flask	y_{out}	–
Molar fraction of oxygen in the liquid	y	–

4. Results & discussion

4.1. Modeling respiration activity and growth of *S. typhimurium*

OTR and growth of *S. typhimurium* TA 100 and TA 98 in the test culture of the Ames RAMOS test were modeled as described in Section 3. The model should qualitatively predict growth and OTR in the test culture to elucidate the phenomena taking place in the test and to determine alternative methods for mutagenicity detection.

With values from Table 2 and equations described in Section 3, biomass growth as well as respiration in the test culture of the proposed Ames RAMOS test was simulated for a negative (non-mutagenic) and positive (mutagenic) control compound, as shown in Fig. 4. Simulated values for strain TA 100 are shown on the left, whereas values for strain TA 98 are shown on the right. There are differences between both cultures in terms of their initial biomass concentration (see Section 3) and mutation rate, as indicated in the figure caption. Distinct mutation rates are not available in literature, but the following assumptions can be made: (1) the negative control is assumed to have a smaller mutation rate than the positive control, where not only spontaneous but also induced mutations happen; (2) for strain TA 100, a generally higher spontaneous mutation rate is assumed than for strain TA 98 (McCann et al., 1975). The used mutation rates only indicate example values to predict the general behavior of the cultures but allow no exact quantitative prediction. The simulated OTR for both strains shows an exponential growth phase during the first hour for TA 100 and during the first three hours for TA 98, independent from the exposure to a negative or positive control (Fig. 4A and B). Afterwards, the OTR drops abruptly and then decreases slowly, before it starts to rise again at 13 h for the positive control and at 20 h for the negative control of strain TA 100, and at 18 h for the positive control and at 25 h for the negative control of strain TA 98. Maximum OTR values of approximately 24 mmol/L/h for strain TA 100 and 16 mmol/L/h for strain TA 98 were reached prior to an abrupt drop in all the OTR curves. Positive controls drop after 21 and 24 h, while the negative controls drop after 25 and 29 h, respectively. The OTR is the sum of the respective OURs of the auxotrophic and prototrophic cells, as illustrated in Fig. 4C and D. OUR curves for the histidine auxotrophic cells correspond to the first part of the overall OTR curves (Fig. 4A and B). However, instead of rising again, they sharply drop after 21, 24, 25, and 29 h, respectively. The OUR curves for histidine prototrophic cells slowly start to rise and then correspond to the second part of the OTR curves.

Fig. 4E–H display the corresponding biomass concentrations of histidine prototrophic and auxotrophic cells, shown both linearly and logarithmically. The biomass concentration of histidine auxotrophic cells rises until 0.18 g/L for strain TA 100 and 0.3 g/L for strain TA 98, until the OTR and OUR of the histidine auxotrophic cells drop for the first time after 1 and 3 h, respectively, after which a slight decrease occurs. The biomass concentration of histidine prototrophic cells rises exponentially over time and reaches a maximum of 0.8 g/L for strain TA 100 and 0.5 g/L for strain TA 98 after 21 and 24 h for the positive and 25 and 29 h for the negative control, respectively. Afterwards, biomass concentrations slowly decrease due to cell death. Fig. 4I and J display simulated glucose and histidine concentrations over time. Histidine is depleted when the OTR drops for the first time. Glucose is depleted after 21, 24, 25 and 29 h, respectively which corresponds to the OTR drops seen in Fig. 4A and B.

The first rise of the OTR curve is due to the respiration of growing histidine auxotrophic cells, which are the only cells present at the beginning of the cultivation. This is also indicated by the rise in the OUR and biomass concentration of histidine auxotrophic cells, as the OTR depicts the overall OUR of the auxotrophic and the prototrophic cell populations and correlates with the biomass concentration. In this initial phase, revertants appear due to spontaneous (negative and positive control) and induced mutations (positive control). As soon as histidine is depleted, histidine auxotrophic cells cannot grow anymore. However,

Table 2
Constant values for modeling the Ames RAMOS test.

Constant	Abbreviation	Value	Comment
Death rate	k_d	0.052 1/h	Fitting own experimental data
Initial concentration of histidine auxotrophic cells	X_0	0 g/L	Assumption: No spontaneous revertants formed during preculture
Maximal growth rate	μ_{max}	0.45 1/h	Own experimental data
Mutation rate	m	Varies between cell types	See caption Fig. 3
Monod constant for glucose	K_{Glc}	0.000182 g/L	(Simkins and Alexander, 1985)
Monod constant for histidine	K_{His}	0.000018 g/L	Fitting own experimental data
Yield coefficient for glucose	$Y_{X/Glc}$	0.47 g/g	Own experimental data
Maintenance coefficient	m_{Glc}	0.5 g/g/h if glucose >0, else 0	Fitting own experimental data
Initial concentration of glucose	Glc_0	4.3 g/L	ISO 11350 (ISO, 2012; Reifferscheid et al., 2012)
Yield coefficient for histidine	$Y_{X/His}$	109 g/g	Own experimental data ^a
Initial concentration of histidine	His_0	0.001 g/L	ISO 11350 (ISO, 2012)
Volumetric mass transfer coefficient	$k_L a$	171 1/h	$k_L a = OTR_{max}/p \cdot L_{O_2} \cdot y_{out, max}$
Maximum oxygen transfer capacity	OTR_{max}	32 mmol/L/h	Calculated after (Lattermann and Büchs, 2016) and (Meier et al., 2016)
Molar fraction of oxygen in head-space when maximum oxygen transfer capacity is reached	$y_{out, max}$	0.186	Calculated with OTR_{max} and Eq. (9)
Oxygen solubility in the medium	L_{O_2}	0.001005 mol/L/bar	Calculated after (Wilhelm et al., 1977; Rischbieter et al., 1996; Weisenberger and Schumpe, 1996)
Pressure	p	1.013 bar	–
Standard volumetric aeration rate	q_g	30 sL/L/h	Mass flow of 10 mL/min, 20 mL of liquid medium
Mole fraction of oxygen in supplied gas	y_{in}	0.2095	–
Standard molar volume of ideal gas	V_{mo}	22.414 sL/mol	–
Molecular weight of glucose	M_{Glc}	180.16 g/mol	–
Stoichiometric coefficient for respiration	ν_{O_2}	6	–
Stoichiometric coefficient for respiration and growth	$\nu_{O_2, growth}$	2.49	–

^a Calculated value of 91 g/g, with 55% protein in cell dry weight (Long and Antoniewicz, 2014) and 1.99% histidine in protein (Spahr, 1962).

they still consume glucose for maintenance. This leads to a metabolic change, resulting in a different stoichiometric factor for the consumption of oxygen, as described in Eqs. (13) and (14). This abrupt change results in a kink in the OTR curve. Because strain TA 98 is inoculated with a higher biomass concentration, the culture consumes histidine faster, resulting in an earlier histidine depletion than for strain TA 100.

Since auxotrophic biomass concentration declines in the histidine limitation phase due to cell death (determined by the death rate k_d), the OUR of histidine auxotrophic cells and the OTR also decrease. The shape of the OTR curve is typical for a second substrate limitation (a substrate other than the carbon source is depleted, allowing the cells to respire but not to grow) (Anderlei and Büchs, 2001). Revertants, which are histidine prototrophic cells that develop from histidine auxotrophic cells during the initial growth on histidine, can grow without histidine and continue to grow exponentially during histidine limitation. This is shown in Fig. 4G and H, with the logarithmically plotted biomass concentrations over time. During growth on histidine, more cells become histidine prototrophic and start to replicate exponentially, leading to an increase in the logarithmically plotted histidine prototrophic cell concentration curve that is more than linear. After histidine is depleted, no more cells mutate, but reverted cells continue to grow exponentially, leading to a linear increase of the logarithmically plotted prototrophic cell concentration (indicated by dashed tangent). Every revertant contributes to biomass formation after histidine is depleted. The more revertants are present after histidine depletion, the earlier the biomass and breathing activity of the histidine prototrophic cells visibly increase due to exponential growth. The time until biomass concentration of the histidine prototrophic cells rises after histidine depletion therefore correlates with the number of colonies in the Ames agar plate test, as well as the number of wells with an observed color-change in the Ames fluctuation test. Since more revertants develop in a cultivation with a positive control (containing a mutagenic compound), cell concentration, and therefore OUR and OTR, reach their maximum values earlier than in the negative control, where revertants only appear due to spontaneous mutations. After glucose depletion, cells cannot grow anymore, and the OTRs and OURs abruptly decline. Since strain TA 98 already consumes more substrate during histidine limitation (due to its

higher inoculum volume), the maximum biomass, OUR, and OTR peaks are lower due to the presence of less residual glucose.

The model indicates that it should be possible to determine the mutagenicity of a chemical compound (which leads to an elevated mutation rate) by online monitoring of the OTR. The more mutagenic a compound is, the earlier the OTR should rise after histidine depletion compared to a negative control, where only spontaneous mutations occur. From the time difference of the OTR rise between a negative and positive control, or any chemical test sample, at a specific threshold, the degree of mutagenicity can be determined. With a reasonable threshold of 4 mmol/L/h (see Fig. 4A and B) and the assumed mutation rates, a time difference of ~5 h for strain TA 100 and ~6 h for strain TA 98 between the negative and positive controls is expected in the simulated data.

4.2. Monitoring respiration activity and growth of *S. typhimurium*

Experimental data is needed to confirm the model described above. For verification, a negative and a positive control compound were used, which can easily be compared to results of the Ames fluctuation test. Pure DMSO was used as the negative control compound, and for the positive control compound, either nitrofurantoin (strain TA 100) or 4-nitro-*o*-phenylenediamin (strain TA 98) dissolved in DMSO were used following the concentrations indicated in ISO 11350 (2012) and by Reifferscheid et al. (2012). Online monitoring of the OTR was conducted in duplicates in a RAMOS device, as seen in Fig. 5A and B. OUR measurement of each co-culture is experimentally not possible. Offline flasks, cultivated in parallel, give information about OD, biomass concentration (Fig. 5C and D), and pH value (Fig. 5E and F).

The course of the OTR corresponds to the simulated OTR in Fig. 4A and B, with an initial growth on histidine within the first few hours, the onset of histidine limitation, and visible growth of revertants. The difference between the strains in the initial OTR increase, indicating differences in growth due to differences in initial ODs of the two strains, is even more pronounced than in the model. This could indicate that other factors except initial OD differ between the strains. The kink in the OTR curve after three (TA 100) and one (TA 98) hour upon histidine

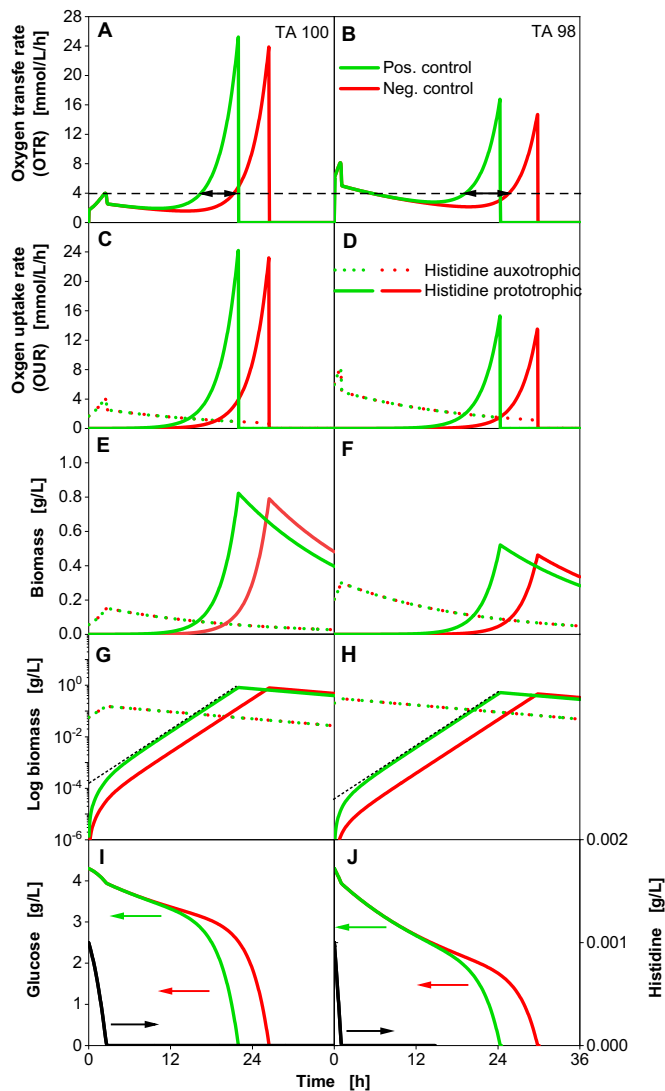


Fig. 4. Simulating respiration activity and growth of *S. typhimurium* TA 100 and TA 98 in the test culture of the Ames RAMOS test, assuming the presence of a negative and a positive control compound. The oxygen transfer rate (OTR), resulting from the sum of oxygen uptake rates (OURs) of auxotrophic and prototrophic cells (A, B), is shown. Time difference at an OTR = 4 mmol/L/h threshold between negative and positive control is indicated by black arrows. The time difference correlates with the mutagenicity of a test compound. The earlier the OTR rises in contrast to a negative control, the more mutagenic is a test compound. Furthermore, OUR for histidine auxotrophic and prototrophic cells (revertants of auxotrophic cells) (C, D), biomass formation of histidine auxotrophic and prototrophic cells (E, F, logarithmic scale: G, H; linear slope indicated by tangent), and glucose and histidine consumption (I, J) were simulated. Initial biomass concentrations used were 0.06 g/L (TA 100) and 0.21 g/L (TA 98). Assumed mutation rates for the negative controls were 0.00008 1/h (TA 100) and 0.000005 1/h (TA 98) due to spontaneous mutations. Assumed mutation rates for the positive controls were 0.0005 1/h (TA 100) and 0.00005 1/h (TA 98). These rates result from a total of spontaneous mutations and mutations induced by a mutagenic test compound. Calculation parameters: 1000 output steps from 0 to 48 h, Runge-Kutta integration method.

depletion, respectively, is not as prominent as in the model. An abrupt change between the two metabolic phases (unlimited and histidine-limited) is assumed in the model. However, the bacteria are obviously able to utilize accumulated histidine within the cells, which leads to a smooth transition. After histidine depletion, the OTR decreases just as assumed in the model. If no cells would lyse during this cultivation phase, the OTR would remain constant. However, since the experimentally obtained OTR decreases, assuming the presence of lysing cells is reasonable to correctly model the OTR course during the Ames RAMOS test. The enhanced growth of strain TA 98 before histidine

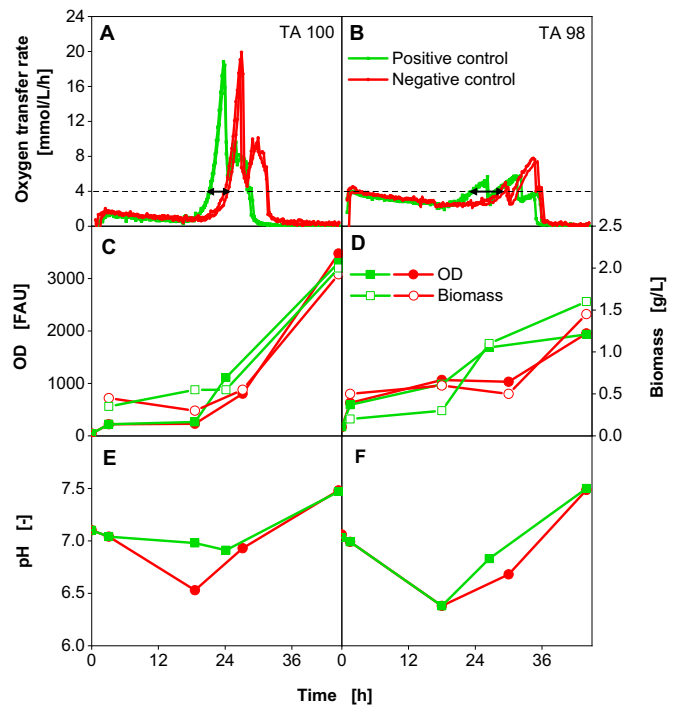


Fig. 5. Results of the new Ames RAMOS test, conducted by online monitoring of the oxygen transfer rate (OTR) with parallel offline sampling during the test culture. OTR was online monitored in duplicates (A, B). Time difference at an OTR = 4 mmol/L/h threshold between controls is indicated by black arrows. The earlier the OTR rises in contrast to a negative control, the more mutagenic is a test compound. Optical density, biomass (C, D) and pH (E, F) were measured at characteristic time points of the OTR curve from shake flasks cultivated in parallel. Cultivation conditions: *S. typhimurium* TA 100 and TA 98, 37 °C, 250 rpm, 50 mm shaking diameter, filling volume 20 mL (250 mL flasks); preculture in RAMOS device: complex medium containing 18.8 g/L nutrient broth powder (Oxoid nutrient broth No. 2), pH₀ = 7.5; test culture in RAMOS device: minimal medium (1.0 × concentrated for TA 100, 1.2 × concentrated for TA 98) containing 1 mg/L histidine, pH₀ = 7.0, OD₀ = 45 ± 5 FAU, negative control: DMSO, positive control: 0.021 μmol/L nitrofurantoin (TA 100) or 130.6 μmol/L 4-nitro-phenylenediamine (TA 98) dissolved in DMSO.

depletion results in more cells respiring on glucose during histidine limitation. This leads to an earlier depletion of glucose when using the standard (1.0 ×) concentrated minimal medium, as shown in supplementary file 2. The glucose peak in the OTR curve for the negative control at 27 h is, therefore, not well-established, impeding mutagenicity detection. Hence, if glucose is depleted too early, revertants cannot grow enough to establish an OTR peak high enough for mutagenicity detection. To generate an OTR peak based on glucose consumption after histidine depletion that is high enough to be reliably detected, 1.2 × concentrated minimal medium was used.

Instead of observing only one OTR peak after histidine depletion as was seen in the simulation, two OTR peaks were visible (Fig. 5A and B). Only one carbon source (glucose) is assumed in the model. However, the experimental medium also contains citrate as a carbon source and complexing agent. This compound is therefore consumed after glucose depletion (Anderlei and Büchs, 2001). However, this is not relevant for the detection of reverted cells and is neglected in the model. Upon glucose consumption, total biomass values of 0.6 g/L (negative and positive control) for strain TA 100, and 0.5 (negative control) and 1.2 g/L (positive control) for strain TA 98 were reached (Fig. 5C and D). Values do not match perfectly with the simulated data but instead fit to the course of the simulated biomass and the experimentally acquired OD. A maximum of 3200 FAU for strain TA 100 and 2000 FAU for strain TA 98 was reached. As earlier discussed, the latter strain consumes more substrate during histidine limitation due to a higher amount of inoculum.

The pH value was measured for comparison with the Ames fluctuation test. Here, pH drops due to growth after histidine limitation. In the Ames fluctuation test, this is detected by the pH indicator bromocresol purple, which changes from purple to yellow when pH reaches a value below 5.2 (Rasheed et al., 2012). In the Ames RAMOS test, pH value drops in all cultures at the beginning of the cultivation from 7 to approximately 6.3–6.9 before rising again to values above 7.3, as shown in Fig. 5E and F. This is due to the initial consumption of NH_3 , leading to a decrease in pH (Meissner et al., 2015; Herweg et al., 2018), followed by the utilization of citrate which then leads to an increase in pH. The pH value does not drop in our experiments to an extent that would cause a clear color change of the pH indicator bromocresol purple (ISO, 2012; Rasheed et al., 2012). Therefore, the pH value must drop further in the Ames fluctuation test than in the Ames RAMOS test. This is most probably due to the fact that microtiter plates used in the Ames fluctuation test are cultivated under oxygen limited conditions under non-shaking conditions (Maier and Büchs, 2001). Under oxygen limited conditions, *S. typhimurium* produces acids (e.g. acetic and formic acid) as anaerobic byproducts, which lead to a pronounced decrease in pH (Wilson et al., 2003). In the Ames RAMOS test, cells are cultivated under oxygen unlimited conditions. It is recommended to ensure that the amount of oxygen available for the used *S. typhimurium* strains has no impact on the spontaneous mutation rate (Mortelmans and Zeiger, 2000). Therefore, cultivations of a negative control of both strains with a volumetric mass transfer coefficient (k_{La}) of 171 1/h and 430 1/h (the k_{La} describes how efficient oxygen is transported from the gas to the liquid phase) are compared as shown in supplementary file 3. For strain TA 100, no difference in the time point of the OTR increase (= number of spontaneous mutants) is observed between the two conditions. Thus, an increased oxygen supply has no negative effect on the amount of spontaneous mutants. For strain TA 98, the OTR of the cultivation with a lower oxygen supply increases slightly earlier than the OTR of the cultivation with a higher oxygen supply. Hence, an increased oxygen supply does not have an oxidative stress-induced negative effect on TA 100 and TA 98. Both strains are apparently not particularly susceptible to excessive aeration and oxidative stress and are, therefore, well-suited for mutagenicity detection in the Ames RAMOS test.

With a chosen OTR threshold of 4 mmol/L/h, time differences of ~4 h for strain TA 100 and of ~6 h for strain TA 98 were detected between the negative and positive control in the experimental data (black arrows in Fig. 4A and B). The practical differences in mutation rates between strains subjected to positive and negative controls are not as large as assumed in the model (see caption of Fig. 4). Nevertheless, data from both the simulation and experiment clearly show the same trend, and the experimentally obtained OTR and biomass curves can be described well by the model. The general principle of the Ames RAMOS test is, therefore, proven.

The obtained results are comparable to results from the Ames fluctuation test performed in 384-well microtiter plates, as seen in Fig. 6. The Ames fluctuation test was conducted with the same batch of control compounds and bacteria. The more wells out of 48 wells that change color after 48 h, the more cells have reverted to histidine prototrophy. Results for the negative controls are compared in Fig. 6A and B. Four and one wells change color for strain TA 100 and TA 98, respectively (Fig. 6A). Hence, the trend for the negative controls, which was observed in the Ames RAMOS test (Fig. 6B), can be verified by the Ames fluctuation test. However, a quantitative comparison of the negative control values was not possible due to the completely different evaluation methods. Fig. 6C and D show differences between negative and positive controls for both test methods. For the Ames fluctuation test, the difference in the number of color-changed wells is shown. For the Ames RAMOS test, the time difference for the OTR rise with a threshold of 4 mmol/L/h, termed as the separation efficiency, is presented. The differences between the two strains using the two test methods can be compared in a quantitative manner. In the Ames fluctuation test, strains TA 100 and TA 98 show a difference of 42 and 46 wells, respectively.

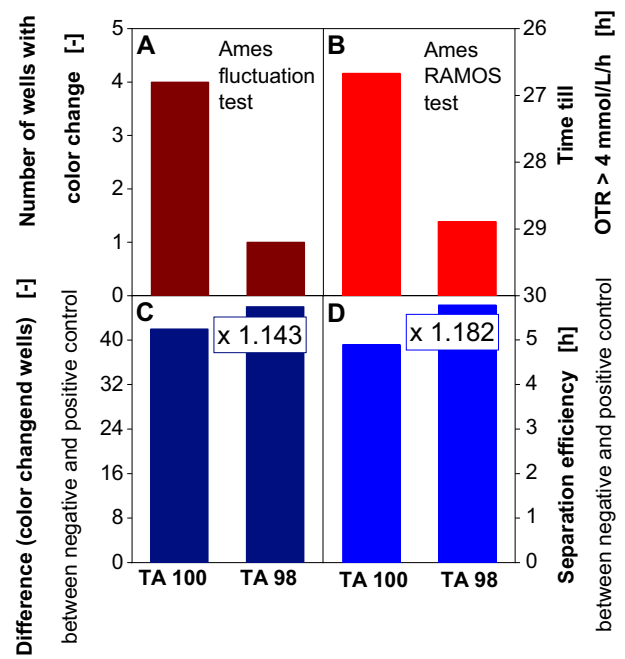


Fig. 6. Comparison of test results of the Ames fluctuation test and the Ames RAMOS test. The Ames fluctuation test was conducted according to ISO 11350 (ISO, 2012) with the same batch of control compounds and bacteria as used in the Ames RAMOS test. Results of negative controls for *S. typhimurium* TA 100 and TA 98 are compared (A, B). Because of a different scaling, only a qualitative comparison can be carried out. Differences in the number of color changed wells (Ames fluctuation assay, C) and the time of OTR rise (Ames Ramos test, D) between negative and positive controls (0.25 mg/L nitrofurantoin for strain TA 100 and 0.01 g/L 4-nitro-*o*-phenylenediamine for strain TA 98) for both strains were compared. The factor by which the difference of strain TA 98 outreaches the one of strain TA 100 is displayed. A mean out of two was calculated for the results of the Ames RAMOS test.

This is equivalent to a ratio of 1.14. In the Ames RAMOS test, the ratio of separation efficiencies for the negative and positive control for strain TA 98 and TA 100 is 1.18. Hence, both test methods show the same trend.

It has to be mentioned that the Ames RAMOS test in this study was conducted in shake flasks with a liquid filling volume of 20 mL. This is not suitable for a high throughput mutagenicity test that generates dose response data. Therefore, an Ames RAMOS test in a microtiter plate format is proposed. The RAMOS technology was transferred to a 48-well microtiter plate format (Flitsch et al., 2016). Table 3 compares the standardized Ames test on agar plates and the Ames fluctuation test with the Ames RAMOS test, showing that the Ames RAMOS test has several advantages. In contrast to standardized test systems, which have a 48 h long incubation time, the Ames RAMOS test can be performed in <48 h since the crucial OTR increase occurs after ~30 h. Additionally, online monitoring of the OTR facilitates an automatic computer-controlled calculation of the OTR increase and, thus, enables evaluation of the test results, without counting colonies or reverted wells. Furthermore, the time-resolved OTR measurement provides an online signal. Unintended deviations in test execution are easily recognized due to online monitoring of the OTR. By online monitoring of the initial OTR increase due to the growth on histidine, the cytotoxicity of a sample can easily be recognized without an extra optical density measurement as in the Ames fluctuation test (ISO, 2012). A decreasing OTR slope during initial growth can indicate possible cytotoxicity, as the OTR slope represents the actual growth rate of the cells. A cytotoxic compound leads to a reduced growth rate. Notably, in the Ames test on agar plates, no additional OD measurement is necessary for cytotoxicity determination (Maron and Ames, 1983). An advantage of the Ames RAMOS test over the Ames fluctuation test, and the Ames luminescence test, as described by Zwart et al. (2018) is the elimination of a separate

Table 3
Comparison of the different Ames test systems.

	Ames agar plate test	Ames fluctuation test	Ames RAMOS test
Duration (without preculture and preparation)	48 h	48 h	~30 h
Evaluation	Counting of colonies	Counting of reverted wells	Automatic computer-controlled calculation and evaluation
Information content	Low (endpoint determination, growth vs. no growth)	Low (endpoint determination, pH decrease and initial OD measurement)	High (time-resolved OTR measurement)
Cytotoxicity detection	Background lawn (no manual intervention)	Initial OD measurement	Initial increase of OTR (no manual intervention)
Workload	High	High	Low (microtiter plate format)
Material consumption	High	Medium	Low (microtiter plate format)
Quantification	Mutants/plate	Reverted wells/overall wells	Time until OTR increase

incubation phase. Because of the time-resolved mutagenicity detection, manual division of the cultures into different wells is not necessary. In the exposition phase of the Ames fluctuation test, each well of a 24-well microtiter plate is distributed into 48 wells prior to the incubation phase. Because this step is not performed in the Ames RAMOS test, a reduced workload is achieved. Moreover, in comparison to the Ames agar plate test, the workload as well as the material consumption is reduced; if the Ames RAMOS test is conducted in microtiter plates, 6 dose response data sets each with 6 concentrations and a negative and positive control can be conducted in one 48-well microtiter plate.

The optimization and downscale of the experimental procedure, including the investigation of reproducibility and generation of dose response data of an Ames RAMOS test in microtiter plates, is described in a subsequent publication.

5. Conclusion

Within the first part of this study, the growth and respiration activity of *S. typhimurium* TA 100 and TA 98 during the test culture of the newly-developed Ames RAMOS test were mechanistically modeled. Co-cultivation of histidine auxotrophic and prototrophic cells was assumed. Respiration of the two strains was seen by two distinct OURs. The sum of the two OURs corresponded to the OTR, which can be experimentally measured. Histidine auxotrophic cells, while growing on initially added histidine, can regain their ability to produce histidine and become histidine prototrophic. The more mutants appeared, the earlier the growth and respiration of the histidine prototrophic culture is visible in the overall OTR course. A time difference in the described rise of the OTR of a negative (non-mutagenic) and positive (mutagenic) control is introduced as a descriptor for mutagenicity.

The simulated data was validated through the online measurement of the OTR in a RAMOS device for shake flasks with both a negative and positive control. A time difference in the rise of the OTR between the two controls could be confirmed.

The comparison of the Ames RAMOS test with the Ames fluctuation test indicated that the general principle behind the Ames RAMOS test is valid. The developed model of a co-cultivation of histidine auxotrophic and prototrophic cells, emerging from auxotrophic cells, appropriately described the phenomena occurring during the Ames RAMOS test. The experimentally obtained OTR curves could be well represented by the model. The evaluation of the time shift between OTR curves from a non-mutagenic and mutagenic sample is a suitable method to detect mutagenicity in the novel Ames RAMOS test. Due to online monitoring, the test gives more detailed information on what is happening during cultivation. Unintended deviations are easily recognized. There is no need to manually count colonies as in the original Ames plate test, or to count the number of color-changed wells as in the Ames fluctuation test. The evaluation of test results can automatically be performed by

the control computer of the RAMOS device. Furthermore, evaluation of the crucial OTR increase takes place after ~30 h at the latest. This means that the test can be performed in <48 h. In future, a μ RAMOS device for online monitoring of OTR in microtiter plates facilitates a high-throughput Ames RAMOS test. The reproducibility, as well as the ability to generate dose response data therefore establishes the Ames RAMOS test as a new mutagenicity test system. These data, as well as the results obtained through the addition of a metabolization system such as S9, is presented in a subsequent publication.

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CRedit authorship contribution statement

Kira Kauffmann: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization. **Lisa Gremm:** Investigation. **Julia Brendt:** Validation, Investigation, Writing - review & editing. **Andreas Schiwy:** Writing - review & editing, Supervision. **Kerstin Bluhm:** Funding acquisition, Conceptualization, Writing - review & editing. **Henner Hollert:** Funding acquisition, Conceptualization, Writing - review & editing, Supervision, Project administration. **Jochen Büchs:** Funding acquisition, Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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