Characterization of enzyme activities and cofactors involved in bioactivation and bioinactivation of chemical carcinogens in the tester strains *Escherichia coli* K12 MX100 and *Salmonella typhimurium* LT2 TA100

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MX100 is an *Escherichia coli* K12 genotoxicity tester strain, especially developed for mechanistic studies of chemical mutagens and carcinogens. For the study of the role of specific enzymes in the bioactivation and bioinactivation of carcinogens, it is necessary to characterize MX100 as far as its metabolic bio(in)activation capacities are concerned. In this study such a characterization is performed in two types of cell-free lysates, one derived from stationary phase cells, grown in rich medium (SR-lysates) and one from exponentially growing cells (log phase), cultured in minimal medium (LM-lysates). Six Phase I enzyme activities of aromatic NADPH hydroxylase, NADH hydroxylase, flavin-containing monoxygenase (FMO), nitroreductase, DT-diaphorase and NADPH ferredoxin:oxidoreductase were determined. Activities of six Phase II enzymes glutathione S-transferases (GSTs), N-aryl acetyltransferase (NAT), arylamine sulphotransferase, UDP-gluconuronyltransferase and epoxide hydratase and of the Phase III enzyme cysteine conjugate β-lyase were subsequently assessed. In addition, five antioxidant enzymes: superoxide dismutase (SOD), catalase, glutathione (GSH)-reductase, GSH-peroxidase and alkyl hydroperoxide reductase; as well as concentrations of glutathione (GSH) and its disulphide (GSSG), were measured. The activity parameters of all enzymes were compared with those obtained in similar lysates of the *Salmonella* strain TA100 and in rat liver preparations. The results indicate that MX100 as well as TA100 contain relatively low oxidative but high reductase Phase I activities. Both strains demonstrated low activities for the Phase II conjugation enzymes except for GSTs. In MX100, relatively high activities were detected for all antioxidant enzymes, activities which were lower in TA100. Significant differences in activities were observed between the SR-lysates derived from stationary phase/rich medium and LM-lysates from log phase/minimal medium cells for nitroreductase, GST, SOD, catalase, NADPH ferredoxin:oxidoreductase as well as in GSH content. In general, we described for the first time a metabolic characterization of the *E.coli* tester strain MX100 and the *Salmonella typhimurium* strain TA100 and discussed the results in terms of its significance for carcinogen bioactivation and bioinactivation capacities.

Introduction

Many chemical carcinogens elicit their biological effects through covalent modifications of DNA (Miller, 1970; Heidelberger, 1975; Singer and Essigmann, 1991). After replication, this process can lead to mutations and ultimately even to cancer. Most chemical carcinogens are relatively inert per se, and require metabolic activation before undergoing covalent interactions with nucleophilic groups in DNA and RNA (Miller and Miller, 1981). The influence of metabolic activation of chemicals into electrophiles and their detoxification has been considered almost exclusively at the level of tumour-initiation (Heidelberger, 1975; Miller and Miller, 1981; Singer and Essigmann, 1991; Guengerich, 1992), although recently it was recognized that mutagenesis is of importance in other stages of carcinogenesis as well (Guengerich, 1992). The majority of the original cell systems used in genotoxicity assays are not metabolically competent or have lost this capacity (Miller and Miller, 1981; Langenbach et al., 1992). The addition of metabolic activation systems to these cell systems has greatly contributed to the success of genotoxicity assays, although addition of such systems still has a number of drawbacks (Bridges and Hubard, 1980; Langenbach et al., 1992). As metabolic activation occurs outside the target cell, the sensitivity of detection of highly reactive, short-lived metabolic intermediates may be limited. Furthermore, metabolic activation systems usually constitute multiple (iso)enzymes which makes it difficult to determine the relative contribution of individual (iso)enzymes. In order to overcome these drawbacks, novel metabolically competent cell systems for the use in genotoxicity testing are being developed (for review see Ruffe et al., 1996).

A great deal of effort has been put into the expression and regulation and into the characterization of mammalian enzymes, involved in the bio(in)activation of chemical carcinogens (Guengerich, 1992; Langenbach et al., 1992). One approach to determine the contribution of specific mammalian (iso)enzymes in the bio(in)activation of chemical carcinogens is heterologous expression of mammalian cDNAs in the target cell (Gonzalez et al., 1991; Langenbach et al., 1992). Recently, we reported on a new *Escherichia coli* K12 genotoxicity tester strain MX100, especially developed for the study of mechanistic aspects of genotoxic carcinogens (Kranendonk et al., 1996). MX100 is an improved derivative of MR2101/pKR11 (Kranendonk et al., 1994) and is more sensitive to a variety of mutagens, including oxidative mutagens which are monitored by back mutation to L-arginine prototrophy via base-substitution mutations. Due to this characteristic and the fact that this strain is derived from the well known *E.coli* K12 laboratory strain AB1157, MX100 is an interesting strain for the development of specialized tester strains in metabolic studies of chemical carcinogens (Kranendonk et al., 1996). Enzyme activities in tester strains can be manipulated by: (i) inactivation or overexpression of specific bacterial genes whose products are involved in the bio(in)activation of carcinogens; or by (ii) heterologous expression of specific mammalian drug-metabolizing enzymes. A prerequisite for the use of MX100 as a mammalian enzyme expressing cell system in genotoxicity
studies, however, is that the cell system itself is fully characterized for endogenous biotransformation capabilities as these could interfere with the bio(in)activation activities of the expressed enzyme and could lead to incorrect conclusions concerning the role of the expressed enzyme in the bio(in)activation of a chemical.

Although bacteria are not generally considered to be metabolically competent (Miller and Miller, 1981; Langenbach et al., 1992), these organisms, being part of the microflora of the gut, saliva and skin, have been implicated in the bioactivation of various chemical carcinogens (Callen, 1978; Laires et al., 1989). Moreover, it has been recognized that some mutagenicity testing bacteria contain specific enzymatic activities which are of importance for the mutagenicity of specific chemicals (McCoy et al., 1983; Kerklaan et al., 1987; Watanabe et al., 1987; Preeto-Alamo et al., 1993).

Therefore, the objective of this study was to characterize the metabolic profile and capacity of the newly developed E. coli K12 tester strain MX100 by which the role of the bacterial metabolism in the (non)mutagenicity of chemicals can be assessed. This characterization consisted of the determination of activities of six Phase I enzymes, six Phase II enzymes, one Phase III enzyme as well as five antioxidant enzymes. Also concentrations of the cofactor glutathione (GSH) and glutathione disulfide (GSSG) were assessed. Activity parameters were compared with those obtained from the Salmonella tester strain TA100 and from rat liver preparations. The metabolic characterization was carried out in cell-free lysates derived from stationary growth phase/rich medium (SR) bacteria and from log phase/minimal medium (LM) bacteria, reflecting the metabolic status of the tester bacterium in two different stages of the test.

Materials and methods

Chemicals and enzymes

L-arginine, GSSG, N-ethylmaleimide (NEM), 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide (CuOOH), acetyl-coenzyme A, 3',5'-adenosine 5'-monophosphate (AMP), nifuroxazide (5-nitro-2-furaldehyde semicarbazone), α-keto-γ-methylbutyric acid (KMB), glutathione reductase (Baker’s yeast) and horse heart cytochrome c were purchased from Sigma Chemical Co (St Louis, MO, USA). Kanamycin, penicillin G, streptomycin and Bacillus subtilis (ATCC 6051) were from Boots Pharmaceuticals, UK. Thiamine, 5,5'-dithiodibis(2-nitrobenzolic acid) (DTNB) and 2,6-dichlorophenolindophenol (DCPIP) were purchased from Merck, Darmstadt, Germany. From other companies were obtained: aniline, nitrobenzene oxide (TOSO) (Aldrich, Steinheim, Germany), pyrogallol and isonicotinic acid (isonicotinic acid hydrizide) (Janssen Chimica), hydroperoxide and thiochrome (JT Baker, Deventer, Holland); Bacto tryptone and Bacto yeast extract (Difco, Detroit, USA); Nutrient broth no 2 (Oxoid, Hampshire, UK), 1,2-epoxy-3-p-nitrophenoxy-propene (EPNP) (Eastman Kodak Co, Rochester, NY, USA) and ferredoxin (spinach, Fluka, Buchs, Switzerland). Thiocyanate and 5-(1,2-dichlorovinyl)-L-cysteine (1,2-DCCV) were synthesized as described previously (Guo and Ziegler, 1991) and Commandeur et al. 1987 respectively.

Bacterial (cell-free) lysates and rat liver preparations

The bacterial lysates of MX100 and TA100 were obtained from cells either overnight grown to stationary phase in rich medium (SR-lyases), under the conditions as used for the two genotoxic tests (Kranendonk et al., 1996 and Maron and Ames, 1983 respectively) or from exponentially growing (log phase) cells (#G Ramos, 0.4–0.6) in standard minimal medium (Maron and Ames, 1983) (LM-lyases). LB-medium was prepared with either 1-arginine (100 µg/ml) and thiamine (1 µg/ml) for MX100 or with L-histidine (54 µg/ml) and #L-histidine (1 µg/ml) for TA100. Cells were incubated at 37°C for 15 min and subsequently harvested by centrifugation at 4000 × g for 10 min at 4°C. All subsequent steps were carried out at 0–4°C. The pelleted cells were resuspended in 100 mM Tris–acetate buffer (pH 7.6), containing 500 mM sucrose and 0.5 mM EDTA resulting in a cell suspension of 70 mg wet weight cells/ml. Following the addition of lysosome (0.2 mg/ml) and an equal volume of water, MX100 and TA100 cells were gently shaken for 10 min. The resulting spheroplasts were pelleted by centrifugation at 4000 g for 10 min and the pellets were resuspended (0.5 g/ml) in 100 mM potassium phosphate buffer (pH 7.4) containing 6 mM magnesium acetate and 0.1 mM dithiothreitol (DTT). The spheroplasts were frozen at –80°C for 1 h and thawed at room temperature in a water bath. MX100 and TA100 cells were lysed by three 30 s bursts of a Labsonic 1510 sonicator while the cells were in an ice bath. The lysates were centrifuged at 10 000 g for 10 min and the resulting supernatants were stored at –80°C until further use.

Rat liver homogenates and microsomal systems were prepared by differential centrifugation of homogenized livers of non-induced male Wistar rats (200–220 g) as previously described (Jefcoate, 1978).

Protein contents of the different bacterial and rat liver preparations were determined according to Ruten et al. (1987), using BSA as standard.

Phase I enzymes

Aromatic (NADH) hydroxylase activity. This was determined by measuring the oxidation of NADH with the substrate aniline as described by Preeto et al. (1993). The reaction mixture contained 10 mM aniline, 0.2 mM NADH and biological sample in a 0.1 M potassium phosphate buffer (pH 8.0) in a total volume of 1 ml. The reaction was started by the addition of NADH and measured spectrophotometrically at 340 nm.

Aromatic (NADPH) hydroxylase activity. This was determined by measuring the formation of p-aminophenol from aniline as described by Imai et al. (1966) with minor modifications. The reaction mixture contained 10 mM aniline, 0.32 mM NADPH, 2.5 mM MgCl 2, 3 mM glucose-6-phosphate, 1.3 U glucose-6-dehydrogenase, 8 mM nicotinamide, 0.1 M potassium phosphate buffer (pH 8.0) and biological sample in a total volume of 1 ml. The reaction was started by the addition of NADPH and terminated after 20 min by the addition of 0.5 ml trichloroacetic acid (20%). After centrifugation, 1 ml of clear supernatant was combined with 0.5 ml NaCO 3 (10%) and 1 ml of phenol (2%) in 0.2 N NaOH. The amount of p-aminophenol formed was determined by measuring the absorption of the indophenol derivative at 630 nm.

DT-diaphorase. This was determined spectrophotometrically at 600 nm by measuring the reduction of 2,6-dichlorophenol-indophenol (DCPIP) as described by Keyes et al. (1984). The reaction medium contained BSA (0.7 mg/ml), 0.2 mM NADH, 0.04 mM DCPIP and sample in a 0.025 mM Tris-HCl (pH 7.4) buffer in a total volume of 1 ml. The reaction was started by the addition of NADH.

Flavin-containing monoxygenase (FMO) activity. This was determined by measuring the thiochrome-dependent oxidation of thiochrome as described by Guo and Ziegler (1991). The reaction medium contained 0.1 M potassium phosphate (pH 7.5), 0.25 mM NADPH, 2.5 mM glucose-6-phosphate, 1.5 U glucose-6-phosphate dehydrogenase, 0.1 U catalase and 0.4 mM EDTA in a final volume of 2.5 ml. After 2 min preincubation, biological sample was added. The reaction was started by the addition of thiochrome (final concentration 1 mM) Aliquots (0.4 ml) withdrawn at 0, 15, 30, 45, 60 and 75 min were added to tubes containing 40 µl of 3 M trichloroacetic acid. Protein was precipitated by centrifugation and 350 µl of clear supernatant was combined with 1 ml of 1.0 M phosphate buffer (pH 7.5), 0.6 ml of water and 50 µl of DTNB (10 mM). The loss of thiochrome was determined as a function of time measuring the absorption at 412 nm.

NADPH-ferredoxin oxidoreductase activity. This was determined by the ferredoxin specific reduction of cytochrome c measured spectrophotometrically at 550 nm (Shin, 1972). The reaction mixture contained (1 ml) contained 50 mM potassium phosphate buffer (pH 7.3), 360 µM cytochrome c, 5.5 mM ferredoxin, biological sample and 1.0 mM NADPH. The reaction was started by the addition of NADPH and the activity was calculated from the initial reaction velocity.

Nitroreductase activity. This was determined spectrophotometrically at 375 nm by measuring the reduction of 5-nitro-2-furaldehyde semicarbazone (nitrofurazone) as described by Watanabe et al. (1988). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM DTT, nitrofurazone (50 µM) and an NADPH generating system (6.7 mM glucose-6-phosphate, 4 U glucose-6-phosphate dehydrogenase, 40 mM NADP + ). The incubations were started by the addition of the biological sample.
Phase II enzymes

Aryl sulphotransferase activity. This was determined spectrophotometrically at 405 nm measuring the release of p-nitrophenol in an incubation mixture (1 ml) containing 10 mM p-nitrophenyl-sulphate (donor-substrate), 20 μM PAP (sulphate mediator) and 0.5 mM phenol (acceptor-substrate) in a 0.1 M Tris-Cl (pH 8.0) buffer as described by Mulder et al. (1977). The reaction was started by addition of the biological sample.

Epoxy hydratase activity. This was determined spectrophotometrically as described by Hasegawa and Hambrock (1982) with the epoxide-stilbene oxide (TSO). The reaction mixture (with a final volume of 1 ml) of both the sample and reference cuvet contained biological sample and 76 mM potassium phosphate buffer (pH 7.4). To the reference cuvet 10 μl ethanol was added leading to a 1% final ethanol concentration. The reaction was started by adding 10 μl of a 5 mM TSO stock solution (in ethanol) to the sample cuvet and the decrease of absorbance was measured at 229 nm.

Glutathione S-transferase activity. This was determined according to Habig et al. (1974) with the substrates CDNB or EPNP. The final reaction mixture (1 ml) contained 1 mM GSH, and either 1 mM CDNB or 1 mM EPNP in a 0.1 M potassium phosphate buffer (pH 6.5). The reaction was started by the addition of biological sample and the increase in absorbance was measured spectrophotometrically at 340 nm (for CDNB) or at 360 nm (for EPNP).

N-acyl acetyl transferase activity. This was determined spectrophotometrically by measuring the formation of the acetylated form of the substrate isonicotinic acid hydrazide (isoniazid) as described by Watanabe et al. (1990). The reaction mixture (90 μl) contained (final concentrations) isoniazid (11.1 mM), acetyl-CoA (2.22 mM) as the acetyl-donor and biological sample in a 50 mM Tris-HCl buffer (pH 7.5). The reaction was started by the addition of the biological sample and terminated after 5 min with the addition of 50 μl trichloroacetic acid (10%). After centrifugation, 112 μl of supernatant was combined with 800 μl of a 0.8 M potassium phosphate buffer (pH 9.0) and the absorbance was measured at 303 nm.

UDP-glucuronosyltransferase activity. This was determined spectrophotometrically according to Fortunato et al. (1986). Incubation mixtures contained biological sample [pretreated for 5 min with 0.25% (v/v) Triton X-100], 5 mM MgCl₂, 1 mM p-nitrophenol and 10 mM UDPGA in a 100 mM Tris-HCl buffer (pH 7.0) buffer in a total volume of 240 μl. The incubation was started by the addition of UDPGA and terminated after 20 min by adding 1 ml of 3% trichloroacetic acid. After centrifugation, 1 ml of supernatant was treated with 5 M KOH (50 μl) and the amount of remaining p-nitrophenol was determined spectrophotometrically at 405 nm.

Phase III enzymes

β-lyase activity. This was determined by a HPLC-fluorescence assay as described by Štěpánková et al. (1992) with minor modifications (Andreadou et al., 1996), using 1,2-DCVC as substrate, measuring the pyruvate release. The incubation mixture contained biological sample (in a final protein concentration of 0.2 mg/ml), 1 mM 1,2-DCVC, 0.1 mM KMB in 50 mM Tris-HCl buffer (pH 8.0) in a total volume of 381 μl. The incubations were started by the addition of sample and terminated after 10 min by addition of 1 ml of an O-phenylenediamine (OPD) solution (12 mM OPD in 3 M HCl). The derivatization of the incubation mixture was performed at 60°C for 60 min. After proteins were precipitated by centrifugation, a 100 μl sample of the supernatant was analysed for the derivatized pyruvate content by HPLC by means of a calibration curve of standardized pyruvate solutions treated equally.

Antioxidant enzymes

Alkyldisperoxiredoxin activity. This was determined spectrophotometrically at 340 nm measuring the NADPH oxidation in the presence of cumene hydroperoxide (Jacobson et al., 1989). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 5 mM EDTA, 3.8 mM NaN₃ (catalase inhibitor), 1.6 mM cumene hydroperoxide, biological sample and 0.3 mM NADPH. The reaction was started by the addition of NADPH and the activity was calculated from the initial reaction velocity. Values were corrected with velocities obtained from the reactions without cumene hydroperoxide.

Catalase activity. This was determined spectrophotometrically at 240 nm by measuring the decomposition of hydrogen peroxide directly as described by Beers and Sizer (1952). The reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.0) biological sample and 5 mM hydrogen peroxide in a total volume of 1 ml. The reaction was started with the addition of hydrogen peroxide.

Glutathione reductase activity. This was determined spectrophotometrically at 340 nm by measuring the oxidation of NADPH in the presence of GSSG as described by Worthington and Rosemeyer (1974). The reaction mixture contained 0.2 M KCl, 1 mM EDTA, 1 mM GSSG and 0.1 M NADPH in a 0.1 M potassium phosphate buffer (pH 7.0) in a total volume of 2 ml. The reaction was started by the addition of biological sample.

Glutathione peroxidase activity. This was determined spectrophotometrically at 340 nm by measuring the oxidation of NADPH in the presence of GSH and H₂O₂ as described by Babson et al. (1981) with minor modifications. The assay solution contained 0.3 mM NADPH, 3.8 mM NaN₃ (catalase inhibitor), 5 mM GSH, 70 mM H₂O₂ and 2 U/ml yeast GSSG-reductase and biological sample in a 50 mM potassium phosphate buffer (pH 7.0) in a total volume of 1 ml. The reaction was started by the addition of 20 μl of a 5.8 mM hydrogen peroxide solution.

Superoxide dismutase (SOD) activity. This was determined by the ability of the enzyme to inhibit the autooxidation of pyrogallol, measured spectrophotometrically at 420 nm as described by Marklund and Marklund (1974). The final reaction volume was 1 ml, containing 50 mM Tris-HCl buffer (pH 8.2), EDTA (1 mM) and pyrogallol (0.2 mM) and biological sample. Activities are expressed in Marklund Units; one unit being defined as the amount of enzyme which causes a 50% inhibition of the pyrogallol autoxidation.

Cofactors

GSH and GSSG content. The cofactor content of the different biological samples was determined by a microtitre plate assay as described by Baker et al. (1990). Briefly, total glutathione (GSH plus GSSG) content was determined via an enzymatic recycling reaction of GSH in a chromogenic reaction with DTNB, finally leading to the formation of 5-thio-2-nitrobenzoate (TNB) with an absorption maximum at 412 nm (Baker et al., 1990). Oxidized glutathione (GSSG) was determined analogously except that reduced GSH was first trapped through reaction with an excess N-ethylmaleimide (NEM).

Results and discussion

We have recently reported on the generation of MX100, a new E.coli K12 tester strain especially developed for mechanistic studies of chemical carcinogens. In the present study, for the first time, a metabolic characterization of MX100 is described by determining 20 different enzyme activities and cofactors, all of potential interest in bioactivation and/or bioinactivation processes of chemical carcinogens. Results were compared with the corresponding data of the S.typhimurium tester strain TA100, analogously determined in this study. A few enzyme activities in certain E.coli strains have been described previously, e.g. SOD and catalase (Prieto-Alamo et al., 1993), glutathione-S-transferases (GSTs) (Arca et al., 1990), nitroreductases (McCalla et al., 1978) and cytochrome β-lyase (Saari and Schultz, 1965). It is well known, however, that differences in enzyme activities can exist between strains, as has been demonstrated for example for different S.typhimurium strains (Meijer et al., 1980). MX100 is a genotoxicity tester strain with specific characteristics (Kranendonk et al., 1994, 1996), which has been obtained following substantial genetic manipulations. These genetic manipulations might in principle have led as well to phenotypical changes of interest.

The MX100 and the Ames mutagenicity test are usually performed by plating cells grown overnight (stationary phase, rich medium) (SR bacteria), in a selective minimal medium. The minimal medium contains a low concentration of the amino acid for which the bacteria are auxotrophic and for which reversion is measured. The limiting concentration of amino acid allows a temporary exponential growth of the tester bacteria (LM bacteria). The composition of the medium of tester plates can substantially influence the mutagenic behaviour of chemicals (Shirai et al., 1980; Thomas and MacPhee, 1987; Kranendonk et al., 1996). Differences in mutagenic response between cells in different growth phases have been shown, e.g. for nitropyrene (Mermelstein et al., 1981).
Fig. 1. Histograms of Phase I, Phase II and Phase III enzyme activities in bacterial lysates of strain MX100 (white bars) and of strain TA100 (shaded bars). LM, lysate derived from cells in the exponential (logarithmic) growth phase, grown in minimal medium. SR, lysate derived from cells in the stationary growth phase, grown in rich medium. *Not determined; **not detectable.

Table I. Phase I enzyme activitiesa in bacterial lysates of MX100, TA100 and in rat liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>MX100a</th>
<th>TA100a</th>
<th>Rat livera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SR-lysate</td>
<td>LM-lysate</td>
<td>SR-lysate</td>
</tr>
<tr>
<td>Aromatic (NADPH)-hydroxylase</td>
<td>Aniline</td>
<td>&lt;3</td>
<td>-</td>
<td>&lt;11</td>
</tr>
<tr>
<td>Aromatic (NADH)-hydroxylase</td>
<td>Aniline</td>
<td>&lt;3</td>
<td>-</td>
<td>&lt;5</td>
</tr>
<tr>
<td>DT-diaphorase</td>
<td>DCPIP</td>
<td>112 ± 5</td>
<td>107 ± 8</td>
<td>162 ± 24</td>
</tr>
<tr>
<td>Flavin containing-monoxygenase</td>
<td>Thiourea</td>
<td>&lt;0.06</td>
<td>-</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Nitroreductase</td>
<td>Nitrofurazone</td>
<td>46.5 ± 0.8</td>
<td>39.3 ± 2.2</td>
<td>35.6 ± 1.5</td>
</tr>
<tr>
<td>NADPH ferrodoxin-oxidoreductase</td>
<td>Cytochrome C</td>
<td>0.4 ± 1.7</td>
<td>62.2 ± 20.7</td>
<td>1.5 ± 1.1</td>
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</table>

*aEnzyme activities are expressed in nmol/min. mg protein of at least duplicate determinations ± SD: <, no activity found, value indicating the lower limit of detectability; -, not determined

The present metabolic characterization of MX100 was therefore carried out in two types of bacterial lysates, namely one derived from cells in the stationary growth phase, grown in rich medium (SR bacteria) and one derived from exponential (log) phase cells, grown in minimal medium (LM bacteria). In this way, a metabolic characterization is obtained of the tester bacteria at the initiation as well as at the first, non-selective, growth stage of the mutagenicity test. The assays in the MX100 strain were carried out in parallel in similar lysates obtained from strain TA100 as well as in rat liver preparations.

Different values for the lower limits of detectability were obtained, e.g. 0.8 and 2.2 nmol/min. mg protein for nitroreductase of the SR and the LM lysate of strain MX100, respectively (see Table I), due to differences in protein concentrations of the four lysates.

Phase I enzymes

Six oxidative and reductive Phase I enzyme activities were determined in strains MX100 and TA100 and in rat liver preparations. The results are summarized in Table I and in Figure 1. Phase I enzymes are in many cases responsible for the first steps in the bio(activation of chemical carcinogens in mammals. The cytochrome P450 and the FMO enzyme systems are considered to be the most important oxidative biotransformation enzymes in this regard (Guengerich, 1992). We have tried to determine cytochrome P450 activity in the bacterial lysates by frequently used mammalian assays by measuring the ethoxycoumarin and ethoxyresorufin dealkylation activities, which are mediated by specific forms of mammalian cytochrome P450 (Lubet et al., 1985). We were not able to determine such activities in the bacterial lysates, however, due to the fact that a metabolism of the products coumarin and resorufin occurred in the SR lysates of MX100 and TA100 (data not shown). Therefore, we were limited to using the less sensitive (NADH and NADPH) p-aniline hydroxylase assay as alternatives for the determination of monoxygenase activities (Ryan and Levin, 1990). The results obtained in the present study indicate that no or only very minor oxidative metabolic activity was present in
MX100 both in SR and LM cells (Table I). This observation is in line with the general view that E.coli do not possess significant cytochrome P450 activity (Smith, 1978). Furthermore, it has been shown that in strain MX100, aflatoxin B1, benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene need an oxidative mammalian metabolizing system for mutagenic activity (Kranendonk et al., 1996). Bacteria nevertheless appear to be able to degrade aromatic hydrocarbons oxidatively to some extent, principally by action of so-called dioxygenases and aromatic hydroxylases (Smith and Rosazza, 1974). A relatively low NADH-dependent aromatic hydroxylase activity in some strains of E.coli has been described previously (Prieto et al., 1993), but this activity could not be measured with aniline in MX100. FMO-activity could not be detected with thiourea as substrate, despite the fact that prokaryotes often possess enzymes that can catalyse the heteroatom oxidation reactions (Guengerich, 1992).

Substantial reductive biotransformation activity was detected in MX100 in the present study. The nitroreductase activity found in MX100 with nitrofurazonine in both SR and LM cells (see Table I and Figure 1) are comparable with the activity found previously in an E.coli B strain (33 nmol/min.mg protein) (McCalla et al., 1970). This reductase activity could be responsible for the mutagenic effect of 4-nitroquinoline-1-oxide and 2-nitrofluorene in MX100 (Kranendonk et al., 1996), as both compounds need bioactivation through nitroreduction (Nagata et al., 1982; Watanabe et al., 1987). In S.typhimurium, this activity has been previously identified as well and was held responsible for the mutagenic effect of nitroarenes in the Ames test (Rosenkranz and Mermelstein, 1983; Watanabe et al., 1989). The nitroreductase activity in TA100, found in the present study is comparable with that found previously in another S.typhimurium, TA1538 (11 nmol/min.mg protein) (Watanabe et al., 1989).

Remarkably high activities of DT-diaphorase or NADH:quinone oxidoreductase were found in the present study in MX100 as well as in TA100, measured with DCPIP. SR and LM lysates of both strains demonstrated activities even higher than in rat liver homogenates. DT-diaphorase is responsible for two-electron reduction of quinones to hydroquinones, a reaction generally considered to be a detoxicating route of quinones (Canadas, 1995), although this enzyme has been implicated in the bioactivation of mitomycin C (MMC) as well (Keyes et al., 1984). MMC did not demonstrate mutagenic activity in strain MX100 (Kranendonk et al., 1996) nor in TA100 (Levin et al., 1982). This mutagenicity, however, is thought to depend on DNA excision repair (Levin et al., 1982) which neither strain MX100 (uvrA) nor TA100 (uvrB) contains. As well as the relation to that in rat liver as well.

Concerning the six Phase II conjugation enzymes measured in MX100 and TA100, significant activities were detected for GSTs conjugating CDNB and UDP-glucuronol transferases conjugating p-nitrophenol (Table II and Figure 1). GST activity towards CDNB was only detected in SR lysates of MX100 and TA100 cells and were similar to the corresponding activities previously reported in stationary cultures of E.coli (3.3 nmol/min.mg protein; Arca et al., 1990) and of Salmonella (5.0 nmol/min.mg protein; Kerklaan et al., 1985). These activities were two orders of magnitude lower than those found in cytosolic fractions of rat liver (Table II). EPNP, a specific substrate for the mammalian O-class GST family (Habig et al., 1974; Ploemen et al., 1995), is considered to represent the ancient progenitor GST of purple bacteria from which the mammalian α-, μ- and π-class GSTs originated (Pemble and Taylor, 1992). However, no enzymatic EPNP GSH-conjugation activity could be detected in any of the lysates of MX100 or of TA100.

In the present study, small but significant N-arylamino acetyl transferases (NAT) activities, conjugating isoniad were detected in SR lysates of both MX100 and TA100 (Table II and Figure 1). Dupret and Grant (Dupret and Grant, 1992) stated that most E.coli strains lack detectable levels of N-arylamino acetyltransferase activities. However, E.coli may contain other types of N-acetyl transferases such as aminoglycoside O-acetyltransferases (Rather et al., 1992). Low NAT activity (2 nmol/min.mg protein) have been shown before with isoniad in lysates of other Salmonella tester strains (Watanabe et al., 1987). In Salmonella tester strains, NAT contains both N-acetyltransferase- and O-acetyltransferase activities (Saito et al., 1985), both shown to be involved in the mutagenicity of aromatic amines (Watanabe et al., 1990).

No significantaryl-sulphotransferase and epoxide hydratase activities could be detected in any of the present bacterial lysates. Regarding the absence ofaryl sulphotransferase activity, this is in agreement with results of Yamozoe et al. (1989), who neither were able to demonstrate such activity with p-nitrophenol in Salmonella strain TA98. E.coli and S.typhimurium, however, have been shown to contain some PAP-dependent sulphotransferase, encoded by cySH and catalysing the transfer to thioredoxin (Ostrowski et al., 1989). With the substrate p-nitrophenol, low activities of UDP-glucuronyltransferase were found in MX100 with no significant differences between and SR and LM lysates (Table II and Figure 1). The activity found in SR lysates of TA100 cells was similar to that found in MX100.

Overall, the results obtained for Phase II enzymes indicate that MX100 like TA100 contains minor Phase II conjugation activities except for GSTs. Compared with rat liver fractions,
Table II. Phase II enzyme activities* in bacterial lysates of MX100, TA100 and in rat liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>MX100*</th>
<th>TA100*</th>
<th>Rat liver*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR-lysate</td>
<td>LM-lysate</td>
<td>SR-lysate</td>
<td>LM-lysate</td>
</tr>
<tr>
<td>Aryl sulphoxidetransferase</td>
<td>p-Nitrophenol sulphate</td>
<td>&lt;0.02</td>
<td>&lt;0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Epoxide hydratase</td>
<td>TSO</td>
<td>&lt;0.3</td>
<td>–</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>GSH-S-transferase</td>
<td>CDNB</td>
<td>6.3 ± 1.8</td>
<td>&lt;1</td>
<td>10.3 ± 1.2</td>
</tr>
<tr>
<td>GSH-S-transferase</td>
<td>EPNP</td>
<td>&lt;4</td>
<td>–</td>
<td>&lt;2</td>
</tr>
<tr>
<td>N-aryl acetyltansferase</td>
<td>Isoniazid</td>
<td>0.6 ± 0.3</td>
<td>&lt;0.7</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>UDP-glucuronyl-transfase</td>
<td>p-Nitrophenol</td>
<td>0.22 ± 0.16</td>
<td>0.64 ± 0.47</td>
<td>0.73 ± 0.36</td>
</tr>
</tbody>
</table>

*Enzyme activities are expressed in nmol/min.mg protein of at least duplicate determinations ± SD; <, no activity found, value indicating the lower limit of detectability; -, not determined.

Table III. Phase III enzyme activities* in bacterial lysates of MX100, TA100 and in rat liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>MX100*</th>
<th>TA100*</th>
<th>Rat liver*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR-lysate</td>
<td>LM-lysate</td>
<td>SR-lysate</td>
<td>LM-lysate</td>
</tr>
<tr>
<td>Cysteine conjugate β-lyase</td>
<td>1,2-DCVC</td>
<td>5.2 ± 2.3</td>
<td>–</td>
<td>8.1 ± 5.6</td>
</tr>
</tbody>
</table>

*Enzymatic activities are expressed in nmol/mg protein of at least duplicate determinations ± SD; -, not determined.

Table IV. Antioxidant enzyme activities* in bacterial lysates of MX100, TA100 and in rat liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>MX100*</th>
<th>TA100*</th>
<th>Rat liver*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR-lysate</td>
<td>LM-lysate</td>
<td>SR-lysate</td>
<td>LM-lysate</td>
</tr>
<tr>
<td>Alkyl hydro-peroxide reductase</td>
<td>CuOOH</td>
<td>1.16 ± 0.44</td>
<td>1.23 ± 0.38</td>
<td>1.33 ± 0.18</td>
</tr>
<tr>
<td>Catalase</td>
<td>H2O2</td>
<td>20.8 ± 0.6</td>
<td>5.8 ± 0.8</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>GSH-peroxidase</td>
<td>H2O2</td>
<td>&lt;7</td>
<td>&lt;8</td>
<td>&lt;5</td>
</tr>
<tr>
<td>GSH-reductase</td>
<td>GSSG</td>
<td>118 ± 15</td>
<td>73 ± 49</td>
<td>297 ± 57</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Pyrogallol</td>
<td>19.5 ± 1.3</td>
<td>6.0 ± 0.9</td>
<td>22.4 ± 0.8</td>
</tr>
</tbody>
</table>

*Enzymatic activities are expressed in nmol/min.mg protein of at least duplicate determinations ± SD; <, no activity found, value indicating the lower limit of detectability; -, not determined.

Toxication of S-substituted L-cysteine conjugates can occur via formation of reactive thiols by the Phase III enzyme S-cysteine conjugate β-lyase (Commandeur et al., 1995). The activities for S-cysteine conjugate β-lyase in MX100 and TA100 found in the present study (Table III and Figure 1) were higher than those observed in rat liver homogenates and similar to those observed in rat renal homogenates (3.05 nmol/min.mg protein), the latter organ being considered to possess the highest β-lyase content (Commandeur et al., 1995). Bacterial β-lyase has been implicated in the catabolism of L-cysteine conjugates of several xenobiotics in the gastrointestinal microflora (Larson, 1985). E.coli β-lyase activity was previously demonstrated to be able to cleave DCVC (Saari and Schultze, 1965), but not S-cysteinyl-N-isopropylacetanilide or S-(2-benzothiazolyl)-cysteine (Larson, 1985).

Antioxidant enzymes
For many chemical carcinogens reactive oxygen species (ROS) are thought to play a role in mutagenesis and carcinogenesis (Frenkel, 1992). Catalase, SOD and GSH-reductase are generally considered to form the bulk of the enzymatic antioxidant activity in the cell. In mammals, GSH-conjugates may undergo further metabolism, resulting in thioether metabolites, such as mercapturic acids (S-substituted N-acetyl-L-cysteines) and the corresponding L-cysteine conjugates (Commandeur et al., 1995).
Characterization of enzyme activities and cofactors in E.coli and S.typhimurium

Fig. 2. Histograms of antioxidant enzyme activities and cofactor contents in bacterial lysates of strain MX100 (white bars) and of strain TA100 (shaded bars). LM, lysate derived from cells in the exponential (logarithmic) growth phase, grown in minimal medium; SR, lysate derived from cells in the stationary growth phase, grown in rich medium. *Not determined; **not detectable.

Table V. Cofactor content in bacterial lysates of MX100, TA100 and in rat liver

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Substrate</th>
<th>MX100</th>
<th>TA100</th>
<th>Rat liver</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SR-lysat</td>
<td>LM-lysat</td>
<td>SR-lysat</td>
<td>LM-lysat</td>
</tr>
<tr>
<td>GSH</td>
<td>DTNB + NEM</td>
<td>35.4 ± 0.6</td>
<td>14.1 ± 0.3</td>
<td>27.2 ± 0.3</td>
</tr>
<tr>
<td>GSSG</td>
<td>DTNB</td>
<td>0.44 ± 0.04</td>
<td>0.51 ± 0.04</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>GSH + GSSG</td>
<td>DTNB</td>
<td>35.9 ± 0.6</td>
<td>14.6 ± 0.3</td>
<td>17.7 ± 0.8</td>
</tr>
</tbody>
</table>

*Contents are expressed in nmol/mg protein of at least duplicate determinations ± SD; -, not determined.

**Deduced by subtracting GSSG content of (GSSG + GSH) content.

*In cell-free homogenate.

alkyl peroxides, Salmonella contains an alkyl hydroperoxide reductase which is more active towards bulky alkyl hydroperoxides (Jacobson et al., 1989). No significant GSH-peroxidase activity could be detected with hydrogen peroxide in any of the lysates of MX100 and TA100 cells, which is in agreement with results from Farr and Kogoma (Farr and Kogoma, 1991). Moreover, the lack of GSH-peroxidase activity in the present bacterial lysates corroborates with the observed lack of activity of GST θ, as measured with EPNP as substrate (see above) (Commandeur et al., 1995). In both SR- and LM-lysates of MX100 cells, a high activity of GSH reductase was detected (Table IV and Figure 2). All these activities were substantially higher than those observed in rat liver homogenates.

For two antioxidant enzymes, MX100 showed significantly different activities when compared with TA100. Catalase activity was substantially higher and GSH-reductase was significantly lower in MX100 when compared with the corresponding lysates of TA100. For SOD and catalase, significant differences were also detected between the LM- and SR-lysates in both strains, demonstrating ~5-fold lower levels in the LM lysates. SR-lysates of both strains showed SOD activity in bacterial lysates of strain MX100 (white bars) and of strain TA100 (shaded bars). LM, lysate derived from cells in the exponential (logarithmic) growth phase, grown in minimal medium; SR, lysate derived from cells in the stationary growth phase, grown in rich medium. *Not determined; **not detectable.

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activities similar to those found in rat liver homogenates. However, all MX100 and TA100 preparations showed lower catalase activities relative to rat liver.

Cofactors
Glutathione concentrations were determined in both the reduced thiol- (GSH) and the oxidized disulphide (GSSG) form in the bacterial lysates and the results are presented in Table V and in Figure 2. The GSH-content in SR lysates of MX100 and TA100 measured in this study were similar to the ones described previously in other E.coli and Salmonella strains (40.5 nmol/mg protein (Romero and Canada, 1991) and 16.7 nmol/mg protein (Kerklaan et al., 1983) respectively). In MX100, we measured a low GSH-content in the LM-lysate and a high content in the SR-lysate. In TA100, this was the opposite. A growth phase dependency of GSH contents in E.coli has been described before (Apontoweil and Berends, 1975). The opposite results in GSH- and GSSG-contents in MX100 and TA100 cannot be explained by differences in GSH-reductase activities, as both strains showed similar activities in both growth phases. The different GSH- and GSSG-contents resulted in a low total GSH-content in log phase MX100 cells with a 2-fold increase in stationary phase cells. Again, this was the opposite in TA100. These results indicate that there is probably an opposite regulation of GSH-homeostasis in MX100 and TA100.

E.coli has been considered unique in the sense that the levels of GSH present in this organism, are comparable to those in mammalian cells. E.coli has therefore been proposed as a suitable model system for oxidant stress in mammalian hepatocytes (Romero and Canada, 1991). In the present study, we showed that this is also the case for MX100 when compared with GSH-levels in rat liver (Moron et al., 1979). It has been shown before that S.typhimurium is less resistant to oxidative stress than E.coli (Farr and Kogoma, 1991). Salmonella strain TA102 showed an overall higher sensitivity towards oxidative mutagens when compared with MX100 with compounds such as hydrogen peroxide, 1-butylhydroperoxide, potassium superoxide or methylglyoxal (Kranendonk et al., 1996). The differences described here in antioxidative enzymes and glutathione contents could explain these characteristics.

In conclusion, in this study we described for the first time a metabolic characterization of the novel E.coli tester strain MX100 and demonstrated that it contains significant carcinogen bioactivation and bioinactivation capacities. Several of these activities could explain the mutagenic behaviour of some compounds, previously demonstrated to respond in strain MX100 (Kranendonk et al., 1996). MX100 showed significant metabolic activities of Phase I enzymes (nitrateductase, DT-diaphorase), Phase II enzymes (GST, NAT, UDP-glucuronyl transferases), Phase III enzymes (cytochrome conjugate β-lyase) and antioxidant enzymes (SOD, catalase, GSH-reductase, alkyl hydroperoxide reductase) and it contained appreciable levels of GSH. The activities found were comparable to those measured analogously in TA100, except for catalase (higher in MX100), GSH-reductase (lower in MX100) and GSH concentrations which were higher and lower in SR- and LM-lysates of MX100 respectively. When compared with rat liver, MX100 as well as TA100 showed negligible Phase I oxidative but high reductive activities, low Phase II activities, (except for GSTs) and substantial levels of antioxidant enzyme activities. Similar activities of the Phase III enzyme cytochrome conjugate β-lyase were found in both MX100 and TA100 and they were comparable to rat kidney levels. Furthermore, a significant NADPH-ferredoxin:oxygen reductase activity was detected in MX100, which is most likely able to support the activity of heterologously expressed mammalian cytochrome P450s, an application for which MX100 seems to be especially appropriate (Kranendonk et al., 1996).

Significant differences in several enzyme activities between the lysates derived from stationary phase/rich medium (SR) and log phase/minimal medium (LM) cells were found in MX100 cells and in TA100 cells. This finding is of importance when the mutagenic or non-mutagenic behaviour of chemicals in these bacterial tester strains is interpreted in terms of bacterial enzyme activities.

Acknowledgements
We are grateful to Dr Bruce N Ames for supplying the Salmonella typhimurium tester strain TA100. M.Kranendonk holds a Ph.D fellowship of the PRAXIS XXI Programme (Portugal). This work was partly supported by the European Community Sub-programme Science and Technology of the 2nd Support Framework

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