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IDENTIFICATION OF *ESCHERICHIA COLI* BY DETECTION OF HYDROQUINONE AND URACIL IN THE URINE SYSTEM

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SUMMARY

For rapid identification of *Escherichia coli*, changes of urinary metabolites incubated with *E. coli* were investigated by gas chromatography—mass spectrometry. Hydroquinone and uracil were detected and the normal urinary constituent 4-deoxythreonic acid was found to diminish in urine incubated with *E. coli*. Hydroquinone could not be detected in urine incubated with *Klebsiella pneumoniae*, *Serratia marcescens* or *Pseudomonas aeruginosa*. Although uracil was detected in normal urine, urine incubated with *E. coli* showed an increased uracil level. Urine incubated with *K. pneumoniae*, *S. marcescens* or *P. aeruginosa* evidenced no such change. A decrease of 4-deoxythreonic acid was noted in urine incubated with *S. marcescens* or *P. aeruginosa*.

In $7.0 \cdot 10^7$ cells of *E. coli*, 0.33—2.36 μg of hydroquinone and 13.4—42.0 μg of uracil were detected after 3 h of incubation at 38°C, and production was not changed after 4, 5 or 8 h of incubation. These results suggest that the detection of hydroquinone and uracil in urine is useful for rapid identification of *E. coli*.

INTRODUCTION

Gas chromatography—mass spectrometry (GC—MS) has been used to identify specific metabolites produced by microorganisms. The detection of the

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compounds by GC or headspace GC has been used as an aid in rapid identification of microorganisms. Cox and Parker [1] reported that 2-aminoacetophenone is the compound responsible for the grape odour in culture of *Pseudomonas aeruginosa* by MS. Labows et al. [2] studied volatile metabolites of *P. aeruginosa* and related species trapped in tubing containing Tenax GC, and a series of peaks representing odd-carbon methyl ketones (2-nonanone and 2-undecane) and 2-aminoacetophenone were identified by GC—MS. Elsden et al. [3] reported that *Clostridium difficile* produced *p*-cresol as an end-product of tyrosine metabolism. They suggested that *p*-hydroxyphenylacetic acid was the precursor of *p*-cresol in the metabolic pathway, and Phillips and Rogers [4] reported that the detection of *p*-cresol by GC provides presumptive identification of this species. Harper and Gibbs [5] found isobutyronitrile, isobutyraldoxime-O-methyl ester and methacrylonitrile in headspace above cultures of *Aeromonas* spp. and *Moraxella* spp. by GC—MS. The availability of 6-hydroxynicotinic acid for rapid identification of *P. aeruginosa* and *Serratia marcescens* was shown in the previous report [6].

In the present study the changes of urinary metabolites before and after incubation with *Escherichia coli* were investigated by GC—MS. Hydroquinone and uracil were found to be the specific metabolites produced by *E. coli* and the detection of hydroquinone and uracil is noted to be clinically useful for rapid identification of *E. coli*.

EXPERIMENTAL

Reagents

Hydroquinone, uracil, hydroxylamine·HCl and *p*-*n*-amylbenzoic acid (ABA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Gasukuro Kogyo (Tokyo, Japan). All other reagents were of the highest purity available commercially.

Bacteria and culture conditions

The bacteria used in this study were *E. coli* strains E101, 102, 105, 106, 107, 108, 111, 112, 113 and 114, *Klebsiella pneumoniae* strain K105, *S. marcescens* strain S108, and *P. aeruginosa* strain P102, which were isolated from urine samples of patients suffering from urinary tract infections and identified by conventional biochemical methods [7]. Each of the bacteria, approximately 10^9 cells of *E. coli* strain E102, *K. pneumoniae* strain K105, *S. marcescens* strain S108, or *P. aeruginosa* strain P102, was inoculated into 8 ml of Casein soy peptone agar (Nissui Pharmaceutical, Tokyo, Japan) and incubated at 38°C for 16 h. After incubation, each media was centrifuged at 1250 *g* at 4°C for 15 min. The supernatant was removed and bacteria were washed twice with 5 ml of sterilized saline and then suspended in 5 ml of sterilized saline.

Viable counts were performed on a bromthymol blue lactose agar (Nissui Pharmaceutical) plate and incubated at 37°C for 24 h. The colonies numbered between 10 and 200.

Urine excreted from a healthy man was immediately sterilized by filtration through a filter with a 0.45-μm pore size (Toyo Roshi, Tokyo, Japan). A 1-ml volume of the sterilized urine was incubated with each of the bacteria and 2 ml

of 0.2 *M* potassium dihydrogen phosphate—disodium hydrogen phosphate buffer (pH 7.0) at 38°C for 3 h.

Sampling and analysis procedure

After incubation, culture media were centrifuged at 1250 *g* for 15 min and 1 ml of the supernatant was taken to form oximes by adding 200 μl of hydroxylamine solution (70 mg/dl) at 60°C for 30 min. After cooling, each of the specimens to which 10 μg of ABA (internal standard) had been added were acidified to pH 1 with 6 *M* hydrochloric acid and saturated with sodium chloride. Acidic compounds were extracted twice with 3 ml of ethyl acetate for 10 min, dehydrated over anhydrous sodium sulphate and evaporated to dryness with a rotary evaporator and a stream of nitrogen. Then the extracts were trimethylsilylated with 100 μl of BSTFA at 65°C for 30 min and 1 μl of the sample was subjected to GC—MS.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a 5710A gas chromatograph (Hewlett-Packard) equipped with a 30-m OV-101 capillary column, a JMS D-300 mass spectrometer (JEOL) and a JMA 2000 data processing system (JEOL). The column temperature was programmed from 100 to 260°C at 3°C/min. Electron-impact (EI) ionization mass spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 300 μA, ion source temperature 200°C and accelerating voltage 3 kV.

Quantitative determination of hydroquinone and uracil

The calibration curve for hydroquinone was obtained using standard solutions with concentrations ranging from 100 ng/ml to 5 μg/ml of phosphate buffer. The calibration curve for uracil was obtained using standard solutions with concentrations ranging from 1 to 50 μg/ml of phosphate buffer. After addition of 10 μg of ABA (internal standard), hydroquinone and uracil were extracted using the same procedure as with the urine sample. Calibration curves relating the concentrations of hydroquinone and uracil were obtained from the mass chromatogram. Ion *m/z* 239 (M^+−15) was used for the quantitation of hydroquinone, ion *m/z* 241 (M^+−15) for uracil and ion *m/z* 264 (M^+) for the monitoring of the internal standard, ABA.

Recoveries of hydroquinone and uracil

Five-fold estimations of hydroquinone and uracil were carried out using 2 μg of hydroquinone and 20 μg of uracil per ml of phosphate buffer. Hydroquinone and uracil were extracted using the same procedure as with the urine sample and quantitated by mass chromatography. Recoveries of hydroquinone and uracil were 41.7 ± 5.3% (mean ± S.D., $n = 5$) and 19.6 ± 3.0%, respectively.

RESULTS

Fig. 1a—d shows typical gas chromatograms of trimethylsilylated derivatives of ethyl acetate extracts from urine incubated with (a) *E. coli* strain E102, (b) *K. pneumoniae* strain K105, (c) *S. marcescens* strain S108 and (d) *P. aeruginosa* strain P102; Fig. 1e shows a control urine.

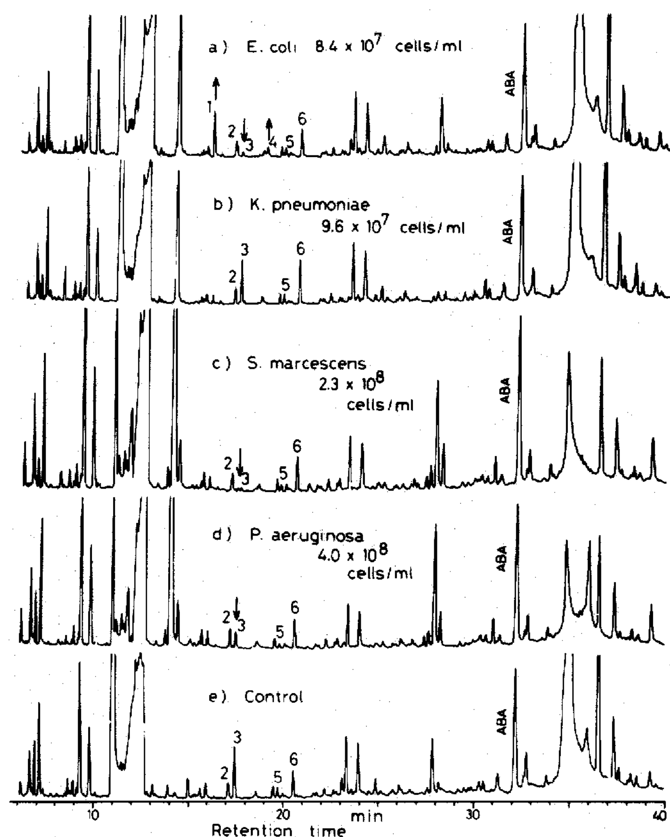

Fig. 1. Gas chromatograms of trimethylsilylated derivatives of ethyl acetate extracts from urine incubated with: (a) *E. coli* strain E102, (b) *K. pneumoniae* strain K105, (c) *S. marcescens* strain S108 and (d) *P. aeruginosa* strain P102, at 38°C for 3 h. Peaks: 1 = uracil; 2 = 4-deoxyerythronic acid; 3 = 4-deoxythreonic acid; 4 = hydroquinone; 5 = 3-deoxytetronic acid; 6 = 2-deoxytetronic acid; ABA = *p-n*-amylbenzoic acid (internal standard).

TABLE I

SPECIFIC COMPOUNDS IDENTIFIED IN INCUBATED URINE

Peak no.	Compound	Mass spectrum of trimethylsilylated derivative*
1	Uracil	256 (51%, M^+), 241 (100), 147 (51), 126 (12), 113 (18), 99 (63), 73 (49)
3	4-Deoxythreonic acid	321 (6%, M^+-15), 292 (38), 220 (17), 203 (9), 147 (43), 117 (72), 73 (100)
4	Hydroquinone	254 (76%, M^+), 239 (100), 223 (4), 112 (8), 73 (31)

*Ionizing energy was 70 eV and ionizing current was 300 μA.

Peak 4 was recognized in urine incubated with *E. coli*, but could not be detected in urine incubated with *K. pneumoniae*, *S. marcescens* or *P. aeruginosa*. The EI mass spectrum of peak 4 is shown in Table I. Peak 4 was identified as trimethylsilylated hydroquinone, because peak 4 and trimethylsilylated hydroquinone showed identical retention times and identical mass spectra.

Peak 1 was found to increase in urine incubated with *E. coli*, but was not changed in urine incubated with *K. pneumoniae*, *S. marcescens* or *P. aeruginosa*. The EI mass spectrum of peak 1 is shown in Table I. Peak 1 was identified as trimethylsilylated uracil, because peak 1 and trimethylsilylated uracil showed identical retention times and identical mass spectra.

Peak 3 was decreased in urine incubated with *E. coli*, *S. marcescens* or *P. aeruginosa*, but was not changed in that of *K. pneumoniae* after incubation. The EI mass spectrum of peak 3 is shown in Table I. Peak 3 was identified as 4-deoxythreonic acid by comparison with the mass spectrum cited in the report of Thompson et al. [8], who characterized 4-deoxythreonic acid, 4-deoxyerythronic acid, 3-deoxytetronic acid and 2-deoxytetronic acid as normal urinary constituents. Peak 2 was identified as 4-deoxyerythronic acid, peak 5 as 3-deoxytetronic acid and peak 6 as 2-deoxytetronic acid [8]. These compounds were not changed after incubation with any bacteria.

Fig. 2 shows one example of the effect of incubation time on the production of hydroquinone and uracil and the decrease of 4-deoxythreonic acid. Sterilized urine (1 ml), to which 2 ml of the phosphate buffer were added, was incubated with $6.0 \cdot 10^7$ or $6.0 \cdot 10^6$ cells of *E. coli* strain E102, at 38°C for 1, 2, 3, 4, 5 or 8 h. The ratios of peak heights of hydroquinone, uracil or 4-deoxythreonic acid, ion *m/z* 292 (M^+−44) and ABA on the mass chromatogram of each case were obtained and the production of hydroquinone and uracil in each

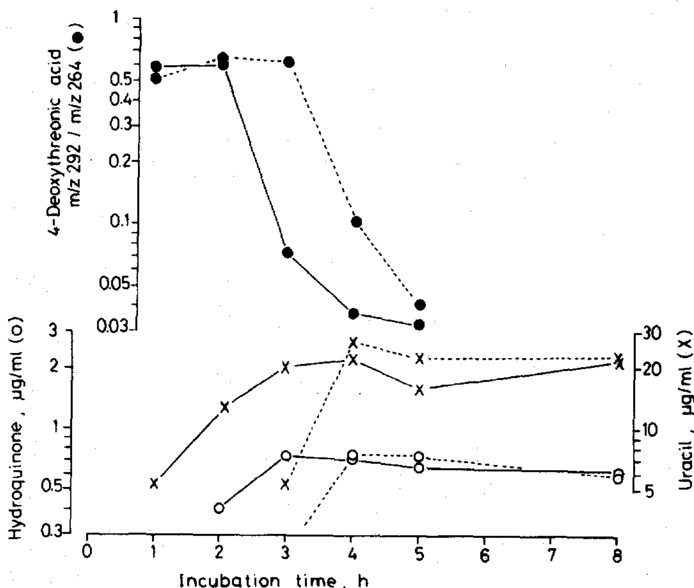

Fig. 2. The effect of incubation time on the production of hydroquinone and uracil and the decrease in 4-deoxythreonic acid in relation to the growth of *E. coli*. (——) $6.0 \cdot 10^7$ cells of *E. coli* strain E102. (---) $6.0 \cdot 10^6$ cells of *E. coli* strain E102.

case was quantitated by calibration curves. Among $6.0 \cdot 10^7$ cells of *E. coli*, 5.0 μg of uracil could be detected after 1 h of incubation, 0.41 μg of hydroquinone and 13.5 μg of uracil after 2 h of incubation and 0.72 μg and 21.5 μg after 3 h of incubation. However, the amount of production after 4, 5 or 8 h of incubation was not changed compared with those after 3 h of incubation. Among $6.0 \cdot 10^6$ of the same cells, although uracil and hydroquinone could not be detected after 1 or 2 h of incubation, 5.2 μg of uracil could be detected after 3 h and 0.75 μg of hydroquinone and 26.0 μg of uracil after 4 h of incubation and the amount of production after 5 h or 8 h did not increase compared with those after 4 h of incubation. The decrease of 4-deoxythreonic acid was recognized after 3 h of incubation among $6.0 \cdot 10^7$ cells and after 4 h of incubation among $6.0 \cdot 10^6$ cells. Among $6.0 \cdot 10^7$ cells of *E. coli*, 3 h of incubation was sufficient to produce hydroquinone and uracil. These results were confirmed by use of other urine.

Table II shows the production of hydroquinone and uracil in six normal urine samples incubated with $7.0 \cdot 10^7$ cells of *E. coli* strain E102 at 38°C for 3 h and the ratios of 4-deoxythreonic acid and internal standard ABA before and after incubation. Hydroquinone and uracil were detected in all specimens after incubation. The production of hydroquinone was 0.33—2.36 μg and that of uracil was 13.4—42.0 μg. The ratios of 4-deoxythreonic acid and internal standrad ABA in every case were decreased after incubation, and the ratios before incubation were 0.12—0.84.

TABLE II

CONCENTRATIONS OF HYDROQUINONE AND URACIL IN SIX NORMAL URINE SAMPLES, AND THE RATIOS OF 4-DEOXYTHREONIC ACID AND INTERNAL STANDARD* BEFORE AND AFTER INCUBATION WITH *E. COLI*

Case	Concentration of hydroquinone (μg/ml)		Concentration of uracil (μg/ml)		4-Deoxythreonic acid*		Creatinine (mg/ml)
	Before	After	Before	After	Before	After	
T.O.	0	1.20	Trace	32.0	0.58	0.04	1.73
O.O.	0	1.10	0	13.5	0.75	0.11	1.03
M.T.	0	0.33	0	13.4	0.12	0.03	0.57
T.T.	0	0.39	0	15.4	0.15	0.04	1.09
H.I.	0	2.36	Trace	42.0	0.84	0.47	1.54
T.N.	0	1.13	Trace	22.4	0.50	0.05	1.37

*Ratios of 4-deoxythreonic acid (*m/z* 292) and internal standard *p-n*-amylbenzoic acid (*m/z* 264) were determined from a mass chromatogram.

The levels of these compounds varied with the urine specimen. Since the variation was considered to be dependent on the concentration of precursors, the amounts of these compounds in diluted urine were estimated. Fig. 3 shows one example of the production of hydroquinone and uracil in diluted urine after incubation with *E. coli*. Sterilized urine was diluted to 4:5, 3:5, 2:5 and 1:5 by the phosphate buffer. Diluted urine or raw urine (1 ml) to which 2 ml of the phosphate buffer were added, was incubated with $6.0 \cdot 10^7$ cells of *E. coli* strain E102 at 38°C for 3 h. The production of hydroquinone or uracil in

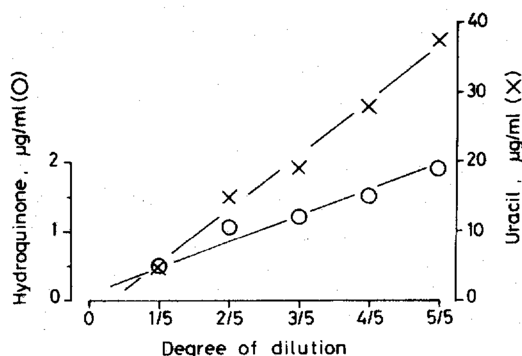

Fig. 3. Concentrations of hydroquinone and uracil in diluted urine incubated with *E. coli.*

TABLE III

CONCENTRATIONS OF HYDROQUIONE AND URACIL IN URINE INCUBATED WITH EACH OF TEN STRAINS OF *E. COLI,* AT 38°C FOR 3 h

Strain	Cell number	Concentration of hydroquinone (µg/ml)	Concentration of uracil (µg/ml)	4-Deoxythreonic acid*
E101	$6.9 \cdot 10^7$	1.65	29.8	0.12
E102	$4.4 \cdot 10^7$	1.60	25.4	0.04
E105	$7.6 \cdot 10^7$	1.70	32.0	0.08
E106	$9.6 \cdot 10^7$	1.78	30.9	0.15
E107	$1.1 \cdot 10^8$	1.70	27.3	0.13
E111	$6.4 \cdot 10^7$	1.48	24.2	0.13
E112	$6.7 \cdot 10^7$	1.48	20.6	0.12
E113	$1.1 \cdot 10^8$	1.70	28.9	0.06
E114	$4.7 \cdot 10^7$	1.05	18.6	0.30
E108	$9.7 \cdot 10^7$	1.70	trace	0.09
Control	0	0	trace	0.53

*Ratios of 4-deoxythreonic acid (*m/z* 292) and internal standard *p-n*-amylbenzoic acid (*m/z* 264) were determined from a mass chromatogram.

each case was quantitated by the above method. After 3 h of incubation, 1.8 µg of hydroquinone and 37.5 µg of uracil were detected in the raw urine. The production of hydroquinone and uracil decreased with the degree of urine dilution, indicating that hydroquinone and uracil originated from urinary metabolites by enzyme reactions.

Since the enzymatic activity of *E. coli* was considered to change from strain to strain, ten strains of *E. coli* were investigated by the above methods. As shown in Table III, all strains showed hydroquinone positive, but one strain showed uracil negative, indicating a possibility of some strain variations.

DISCUSSION

The production of hydroquinone and uracil was recognized in urine-inoculated *E. coli.* The present results indicate that the detection of hydroquinone and uracil in urine is useful for rapid identification of *E. coli.* As

shown in Fig. 2, among 6.0 · 10^7 cells of *E. coli*, 0.72 μg of hydroquinone and 21.5 μg of uracil could be detected and the amounts of hydroquinone and uracil production after 4, 5 or 8 h of incubation were not changed compared with those after 3 h of incubation. Also, as shown in Fig. 3, the amount of hydroquinone or uracil production depends on the urine dilution. These results show that a precursor of hydroquinone or uracil exists in urine. The precursor of uracil is considered to be uridine because a large amount of uracil was detected by adding 2 ml of uridine (500 μg/ml of phosphate buffer, pH 7.0) to urine with *E. coli*, through the action of uridine phosphorylase [9]. The origin of hydroquinone is still not clear, and its production did not increase by adding 2 ml of gentisic acid (100 μg/ml of phosphate buffer, pH 7.0) to urine with *E. coli*, although the action of gentisate decarboxylase was assumed to produce hydroquinone.

4-Deoxythreonic acid and 4-deoxyerythronic acid (diastereoisomers) were characterized as normal urinary constituents by Thompson et al. [8], who reported that urinary excretion of 4-deoxythreonic acid was 15—46 μg/mg of creatinine, and that of 4-deoxyerythronic acid was 3—8 μg/mg of creatinine. The interesting point is that 4-deoxyerythronic acid was not changed after incubation, whereas 4-deoxythreonic acid was enzymatically metabolized from *E. coli*, reflecting the substrate specificity of enzyme.

The amount of hydroquinone or uracil production and normal urinary concentrations of 4-deoxythreonic acid are seen to depend on the urine specimen as shown in Table II. The production of hydroquinone and uracil and the decrease of 4-deoxythreonic acid in urine incubated with *E. coli* suggest that the detection of hydroquinone and uracil is useful for clinical diagnosis of urinary tract infections.

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