Short Communication

DETECTION OF BACTERIAL GALLATE DECARBOXYLATION BY VISUAL COLOR DISCRIMINATION

RO OSAWA* AND TERRY P. WALSH

*Centre for Molecular Biotechnology, School of Life Science,
Queensland University of Technology, Queensland 4001, Australia*

(Received September 22, 1994; Accepted February 11, 1995)

Gallic acid (GA), one of the trihydroxybenzenoids, occurs naturally as a constituent of plant polyphenols such as tannins and flavonoids. There has been continuing interests in bacterial GA catabolism in the gut, soil, and water ecosystems due to its toxicity (2,12) and potent mutagenicity (4,5) of these compounds.

Bacteria capable of degrading GA include *Klebsiella aerogenes* (3) and *Eubacterium oxidoreducens*, isolated from the rumen (7), *Pelobacter acidigallici* from sewage water (16), *Citrobacter* sp. from soil (17), *Enterococcus faecalis* from rat feces (9), *Streptococcus bovis*, *K. oxytoca*, and *K. pneumoniae* from koala feces (11). An initial step of GA degradation by these microorganisms has been found to be anaerobic decarboxylation of GA to pyrogallol (PY) (1,3,8,11,15). Osawa et al. (11) found that a novel facultatively anaerobic bacterium, which is one of the predominant members of the koala's gut microflora, not only converted GA completely to PY even at the final concentration of 100 mM but also utilized it for growth. Detection of such bacterial GA degradation has so far relied on UV spectrophotometry (16,17), gas chromatography (14), and high-performance liquid chromatography (HPLC) (8,11).

During a series of investigations of bacterial degradation of tannin (10,11), the authors recognized that when GA and PY were oxidized in alkaline solutions, the solutions developed green and orange colors, respectively. Based on this phenomenon, a simple visual reading method for detection of the bacterial GA degradation was developed.

Color development of substrate medium (SM) after alkalization was monitored visually, and then analyzed spectrally. The SM consisted of Wilkins-Chalgren

* Present address and address reprint requests to: Dr. Ro Osawa, Kanagawa Prefectural Public Health Laboratory, 52-2, Nakao-cho, Asahi-ku, Yokohama 241, Japan.

anaerobe broth (Oxoid Ltd., Basingstoke, Hampshire, U.K.; pH 7.1) with various combinations of GA (Sigma Chemical Co., St. Louis, MO, U.S.A.) and PY (Sigma) up to 10 mM. A test tube containing 3 ml of SM was alkalized with equal amounts of saturated NaHCO_3 solution (pH 8.6) and then incubated in air at 37°C . At 15 and 30 min and at 1, 2, and 4 h of incubation, the contents of test tubes were mixed thoroughly before a visual judgement of the color was recorded (Table 1) and its spectrum ($A_{400}-A_{750}$) was read in a scanning spectrophotometer (DMS 200; Varian Pty Ltd., Musgrave, Australia).

Up to 60 min incubation, green coloration, which is typical of oxidized GA, was observed only in the SM-containing GA only (8 and 10 mM), whereas other SM gave light yellow to brown coloration, typical of oxidized PY, regardless of the presence of GA. When incubation exceeded 60 min, development of a green color became apparent in the SM containing a mixture of 8 mM GA and 2 mM PY. Figure 1a-c shows the spectral shift observed in the oxidation of 8 mM of GA, 2 mM of PY, and the mixture of both. Although general absorbance increased with time in the mixtures, the spectral pattern was distinctively different for SM containing 8 mM GA and 2 mM PY: GA had a tropic absorbance peak at 640 nm (Fig. 1a) whereas PY did not (Fig. 1b); the spectral pattern typical of PY was apparent in the 8 mM GA and 2 mM PY mixture at 60 min but this pattern was shifted more toward that of GA after 2 h (Fig. 1c). The spectral patterns of other SMs (i.e. 6 mM GA + 4 mM PY, 4 mM GA + 6 mM PY, 2 mM GA + 8 mM PY, and 10 mM PY) were all identical to that of PY at all incubation times in the present study (data not shown). These spectrophotometric results were consistent with the results of visual reading (Table 1).

GA and PY are easily oxidizable in an alkaline solution to form polymerized compounds of *o*-quinone derivatives, resulting in coloration of the medium although the phenomena involve complex chemical reactions (13). In the present study, the oxidization of 8 mM GA was apparently delayed by more than 1 h with the presence of 2 mM PY in the medium. The evidence suggests that oxygen

Table 1. Color development of alkalized media containing gallic acid (GA), pyrogallol (PY), and mixture of both at various concentrations incubated at 37°C for up to 4 h.

Incubation time	Substrate							
	GA (10)	GA (8)	PY (10)	PY (2)	GA + PY (8) (2)	GA + PY (6) (4)	GA + PY (4) (6)	GA + PY (2) (8)
15 min	LG	LG	LY	LY	LY	LY	LY	LY
30 min	G	G	Y	Y	Y	Y	Y	Y
1 h	G	G	O	O	O	O	O	O
2 h	DG	DG	B	B	G/B	B	B	B
4 h	DG	DG	B	B	G/B	B	B	B

LG, light green; LY, light yellow; G, green; Y, yellow; O, orange; DG, dark green; B, brown; G/B, greenish brown. The numbers in parentheses below each substrate is its concentration in mM in the medium.

Fig. 1. Spectral changes observed during alkaline oxidization of 8 mmol GA (a), 2 mmol PY (b), and the mixture of 8 mmol GA and 2 mmol PY (c).

The oxidizing process was initiated by adding saturated NaHCO_3 (pH 8.6) to the media. Spectra were recorded after 15, 30 min, 1, 2, and 4 h of incubation.

available in the medium is initially consumed by PY rather than GA, thereby suppressing the green coloration of GA. In other words, PY was more easily oxidizable than GA. This in turn suggests that bacterial decarboxylation of GA to PY is visually detected after 1 h alkaline treatment of the sample medium if the bacterium cultured in the SM initially containing 10 mM GA, produced a minimum of 2 mM PY.

To substantiate this view, we inoculated a total of 36 strains of various

Table 2. Comparison of the visual reading method (VR) and the reversed-phase HPLC method (HPLC) for detection of gallic acid (GA) and pyrogallol (PY) in the spent media of 36 bacterial strains.

Bacterial strain		GA concn. (mM) (HPLC) ^a	PY concn. (mM) (HPLC)	Color developed at 1 h incubation (VR)	
<i>Streptococcus bovis</i>	ACM ^b 3539	10.0	—	Green	
	ACM 3540	10.0	—	Green	
	ACM 3542	10.0	—	Green	
	ACM 3543	9.9	—	Green	
	ACM 3544	10.0	—	Green	
	ACM 3547	—	9.9	Orange	
	ACM 3550	10.0	—	Green	
	ACM 3611	—	10.1	Orange	
	ACM 3869	—	9.9	Orange	
	ACM 3870	—	9.9	Orange	
	ACM 3871	—	10.0	Orange	
	ACM 3872	9.9	—	Green	
	ACM 3873	—	10.0	Orange	
	ACM 3874	—	10.0	Orange	
	ACM 3902	—	10.1	Orange	
	ACM 3903	—	10.0	Orange	
<i>Str. equinus</i>	ACM 3541	10.0	—	Green	
	ACM 3545	9.9	—	Green	
<i>Str. sangius</i>	ATCC ^c 10556	10.0	—	Green	
<i>Str. pyogenes</i>	QUT ^d 301	10.1	—	Green	
<i>Diplococcus pneumoniae</i>	QUT 302	10.0	—	Green	
<i>Enterococcus faecalis</i>	QUT 401	10.1	—	Green	
<i>Staphylococcus aureus</i>	ATCC 12598	10.0	—	Green	
	QUT 102	9.9	—	Green	
	QUT 103	10.0	—	Green	
<i>Staph. epidermidis</i>	ATCC 12228	10.0	—	Green	
<i>Enterobacter agglomerans</i>	ACM 1615	9.9	—	Green	
<i>Klebsiella pnemoniae</i>	ACM 3844	<0.1	9.9	Orange	
	ACM 90	<0.1	10.0	Orange	
<i>K. oxytoca</i>	ACM 3843	3.0	7.1	Orange	
<i>Citrobacter freundii</i>	QUT 150	10.0	—	Green	
<i>Yersinia enterocolitica</i>	ATCC 29833	10.1	—	Green	
<i>Serratia marcescens</i>	QUT 151	10.0	—	Green	
<i>Escherichia coli</i>	ACM 845	10.0	—	Green	
	ACM 3841	10.1	—	Green	
Tannin-protein complex degrading bacilli		ACM 3666	—	10.1	Orange

^a Mobile phase, 0.02 M, pH 4.5, citrate-phosphate buffer; 20- μ l injection; 254-nm detector; column, μ Bondapak C18 reversed-phase packing (10 μ m); detection limit, 0.01 mM.

^b ACM, Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, St. Lucia, Brisbane, Australia.

^c ATCC, American Type Culture Collection, Rockville, MD, U.S.A.

^d QUT, our collection, School of Life Science, Queensland University of Technology, Garden Point Campus, Brisbane, Australia.

bacterial genera and species that had been grown on Wilkins-Chalgren anaerobe agar (Oxoid), each in 3 ml of SM containing 10 mM GA, and incubated anaerobically at 37°C for 3 days. It should be noted that the growth of all bacteria tested was not significantly inhibited by the presence of GA at the above concentration. After incubation, the SM was centrifuged at $3,000 \times g$ for 10 min, and sterilized by filtration. The aliquot thus prepared was used for detection of GA conversion to PY. The detection was performed by two different methods; a reversed-phase HPLC method as described by Hoefler and Coggon (6) and the visual reading as described above. A 0.5-ml aliquot was acidified with an equal amount of 0.1 N HCl for the HPLC analysis, and the remaining aliquot was combined with an equal amount of saturated NaHCO₃ solution for the visual reading method. It should be noted that coloration of the sample was read at the 1st hour of incubation.

Table 2 shows the results obtained by the two methods. With the HPLC method, complete conversion of GA of PY was detected for 12 strains and the partial conversion for one strain of *K. oxytoca* ACM 3843, which catabolized 7 mM GA to yield 7 mM PY. The remaining 24 strains did not degrade GA to any form. The results obtained from the visual reading were all consistent with those from HPLC. In this method, the aliquot obtained from the spent medium of *K. oxytoca* ACM 3843 gave a distinct orange color in spite of presence of 3 mM intact GA.

Our visual reading method is a simple, reliable method for detection of GA decarboxylation by bacteria. It does not require sophisticated and expensive analytic equipments, such as a spectrophotometer and an HPLC and would be suitable for large-scale screening tests. It is, however, reported that some anaerobic bacteria (i.e. *Eubacterium oxidoreducens*, *Pelobacter acidigallici*) are capable of degrading PY further to other phenolics such as phloroglucinol and resorcinol (7, 14). In the present study, we did not have access to such bacteria for examination but our supplementary experiment showed that media containing various concentrations (1 to 10 mM) of phloroglucinol or resorcinol were found to give a light to dark blue color when oxidized in saturated NaHCO₃ solution (data not shown). Therefore, care should be taken when any color other than green or orange develops using the present method, which is indicative of possible bacterial PY degradation.

We thank L. Sly and D. Grassick of the Department of Microbiology, University of Queensland, for their generous gift of bacterial strains. We are also grateful to K. Matsumoto of the Institute for Animal Experimentation, School of Medicine, University of Tokushima, for his generous support throughout the study.

REFERENCES

- 1) Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T., and De Eds, F., The metabolic fate of gallic acid and related compounds. *J. Biol. Chem.*, **234**, 3014–3016 (1959).
- 2) Dollahite, J. W., Pigeon, R. F., and Camp, B. J., The toxicity of gallic acid, pyrogallol, tannic acid isolated from *Quercus havardi*, in the rabbit. *Am. J. Vet. Res.*, **23**, 1264–1267 (1962).

- 3) Grant, D. J. W. and Patel, J. C., The non-oxidative decarboxylation of *p*-hydroxybenzoic acid, gentisic acid, protocatechuic acid and gallic acid by *Klebsiella aerogenes*. *Antonie van Leeuwenhoek*, **35**, 325–343 (1969).
- 4) Hardigree, A. A. and Epler, J. L., Comparative mutagenesis of plant flavonoids in microbial systems. *Mutat. Res.*, **58**, 231–239 (1978).
- 5) Hirono, I., Ueno, I., Hosaka, S., Takanashi, H., Matsushima, T., Sugimura, T., and Natori, S., Carcinogenicity examination of quercetin and rutin in ACl rats. *Cancer Lett.*, **13**, 15–21 (1981).
- 6) Hoefler, A. C. and Coggon, P., Reversed-phase high-performance liquid chromatography of tea constituents. *J. Chromatogr.*, **129**, 460–463 (1976).
- 7) Krumholz, L. R. and Bryant, M. P., *Eubacterium oxidoreducens* sp. nov. requiring H₂ or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. *Arch. Microbiol.*, **144**, 8–14 (1986).
- 8) Krumholz, L. R., Crawford, R. L., Hermling, M. E., and Bryant, M. P., Metabolism of gallate and phloroglucinol in *Eubacterium oxidoreducens* via 3-hydroxy-5-oxohexanoate. *J. Bacteriol.*, **169**, 1886–1890 (1987).
- 9) Nakajima, H., Otani, C., and Niimura, T., Decarboxylation of gallate by cell-free extracts of *Streptococcus faecalis* and *Klebsiella pneumoniae* isolated from rat feces. *J. Food Hyg. Soc. Jpn.*, **33**, 371–377 (1992).
- 10) Osawa, R. and Walsh, T. P., Visual reading method for detection of bacterial tannase. *Appl. Environ. Microbiol.*, **59**, 1251–1252 (1993).
- 11) Osawa, R., Walsh, T. P., and Cork, S. J., Metabolism of tannin-protein complex by facultatively anaerobic bacteria isolated from koala feces. *Biodegradation*, **4**, 91–99 (1993).
- 12) Rayude, G. V. N., Kadirvel, R., Vohra, P., and Kratzer, F. H., Toxicity of tannic acid and its metabolites for chickens. *Poult. Sci.*, **49**, 957–960 (1970).
- 13) Salunkhe, D. K., Chavan, J. K., and Kadam, S. S., Dietary Tannins: Consequences and Remedies, CRC Press, Boca Raton, Florida (1989), p. 84–87.
- 14) Samain, E., Albagnac, G., and Dubourguier, H.-C., Initial steps of catabolism of trihydroxybenzenes in *Pelobacter acidigallici*. *Arch. Microbiol.*, **144**, 242–244 (1986).
- 15) Scheline, R. R., Decarboxylation and demethylation of some phenolic benzoic acid derivatives by rat caecal contents. *J. Pharmacol. Pharmaceut.*, **18**, 664–669 (1966).
- 16) Schink, B. and Pfennig, N., Fermentation of trihydroxybenzenes by *Pelobacter acidigallici* gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. *Arch. Microbiol.*, **133**, 195–201 (1982).
- 17) Yoshida, H., Tani, Y., and Yamada, H., Isolation and identification of a pyrogallol producing bacterium from soil. *Agric. Biol. Chem.*, **46**, 2539–2546 (1982).