Increasing the Oxidative Stress Response Allows Escherichia coli To Overcome Inhibitory Effects of Condensed Tannins

Alexandra H. Smith1,2† James A. Imlay,3 and Roderick I. Mackie1,4*

Department of Animal Sciences,1 Division of Nutritional Sciences,4 and Department of Microbiology,3 University of Illinois, Urbana, Illinois 61801, and Animal Nutrition and Animal Products Institute, Irene 0062, South Africa

Received 29 July 2002/Accepted 25 February 2003

Tannins are plant-derived polyphenols with antimicrobial effects. The mechanism of tannin toxicity towards Escherichia coli was determined by using an extract from Acacia mearnsii (Black wattle) as a source of condensed tannins (proanthocyanidins). E. coli growth was inhibited by tannins only when tannins were exposed to oxygen. Tannins auto-oxidize, and substantial hydrogen peroxide was generated when they were added to aerobic media. The addition of exogenous catalase permitted growth in tannin medium. E. coli mutants that lacked HPI, the major catalase, were especially sensitive to tannins, while oxyR mutants that constitutively overexpress antioxidant enzymes were resistant. A tannin-resistant mutant was isolated in which a promoter-region point mutation increased the level of HPI by 10-fold. Our results indicate that wattle condensed tannins are toxic to E. coli in aerobic medium primarily because they generate H$_2$O$_2$. The oxidative stress response helps E. coli strains to overcome their inhibitory effect.

Tannins are polyphenolic compounds, produced by a wide variety of plants, that can be separated into two structural groups. Hydrolysable tannins consist of a central polyol, e.g., glucose, surrounded by several gallic acid units. More complex elagittannins are derived from pentagalloylglucose by oxidative reactions (19). Condensed tannins, or proanthocyanidins, are the most common type of tannin found in forage and browse and consist of polymers of flavonol units (24). Tannins may protect plants from herbivory, increase resistance against pathogens, or protect tissues such as wood against decay (23). Their antimicrobial activity has been the focus of research in many fields: food science, wood science, soil science, plant pathology, pharmacology, and human and animal nutrition (23). The complexity and diversity of tannin structures, as well as the presence of mixtures of phenolic compounds in plants, complicate the study of tannins.

The mechanism(s) by which tannins inhibit bacteria have not been clearly elucidated, but the biological activity of tannins is probably determined to a great extent by the molar content and spatial configuration of the ortho-phenolic hydroxyl groups (24). Studies with green tea catechins, which are monomeric polyphenolics, report that Gram-positive bacteria are more sensitive to their bactericidal effect than gram-negative bacteria (12). These catechins have been shown to disrupt membrane integrity, as they cause leakage from liposomes (12). Their activity was lower in the presence of negatively charged lipids, indicating that the higher resistance of gram-negative bacteria can be explained to some extent by the presence of negatively charged lipopolysaccharides (12). Work on green tea catechins by other authors confirms that membrane damage occurred and that flavonoids such as catechins and epigallocatechin gallate insert or interact in the outer polar zone of lipid bilayers in liposomes (9, 13, 21, 29, 32).

However, dihydroxy phenolic groups can form stable complexes with many metal ions. In the presence of a nonlethal concentration of copper(II), catechins were shown to be bactericidal to gram-negative Escherichia coli, resulting in damage to the cytoplasmic membrane (10). The bactericidal activity of catechin-Cu(II) complexes was lower against the gram-positive Staphylococcus aureus (10). In this case, the mechanism of action is proposed to be due to recycling redox reactions between catechin-complexed Cu(II) and Cu(I) that are caused by molecular oxygen, generating hydrogen peroxide on the cell surface (11). The possible reason for a lower activity against gram-positive cells may be that the catechin-copper ion complex does not bind to S. aureus cells, but it appears to be attracted to the negative charge of E. coli lipopolysaccharide, generating hydrogen peroxide locally on the cell surface (10). There are other indications that growth of E. coli may be inhibited by tannins through complexation with metal ions. Growth of E. coli ATCC 25922 on low-iron plates with tannic acid, a hydrolyzable tannin, occurred only around wells containing an iron solution (4). Another experiment indicated that growth of E. coli ATCC 25922 on tannic acid-containing medium was restored after iron addition (4). Increased iron sequestration may therefore prevent inhibition by tannins.

E. coli cells possess antioxidant enzymes which are induced in response to oxidative stress. The oxyR gene positively induces the expression of at least nine hydrogen peroxide-inducible proteins, including hydroperoxidase I (HPI) (7). HPI is an OxyR-inducible catalase encoded by the katG gene expressed during exponential growth, whereas HPII is encoded by katE and induced by RpoS in stationary phase (6). Inducing the H$_2$O$_2$ stress response increased the survival rate of E. coli incubated with catechins in the presence of Cu(II) (11). It seems possible, then, that E. coli could resist the toxicity of tannins by engaging high-affinity iron acquisition systems...
and/or by activating its oxidative stress response. There is variation among *E. coli* strains in sensitivity to green tea catechins, as 8 of 20 clinical *E. coli* isolates were found to be sensitive to the effect of green tea catechins (34).

The present study was done to determine if more complex condensed tannins have an effect on *Escherichia coli* similar to that of green tea catechins. A commercially available extract from *Acacia mearnsii* (Black wattle) used in the tanning industry was used as a source of condensed tannins. The tannins present in this extract range from 300 to 3,000 atomic mass units, with an average numerical mass of approximately 1,250 atomic mass units. Our approach was to employ an *E. coli* strain that was sensitive to the effect of tannins and isolate spontaneous tolerant mutants. In this way, we could detect the mutation that confers the resistance and deduce the nature of the stress caused by tannins. This paper reports our findings for auto-oxidation of tannins resulting in hydrogen peroxide production inhibits growth of *E. coli*. An increase in the oxidative stress response in *E. coli* can decrease the inhibitory tannin effect.

**MATERIALS AND METHODS**

**Strains.** *E. coli* strains used in this study are listed in Table 1.

**Media.** Experiments were performed on MOPS medium (20) with glucose (0.4%) as the carbon source and iron and trace elements provided by trace element solution SL-10 (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Medium without glucose was prepared anaerobically was gassed with CO2 and incubated at 37°C.

**Culture conditions and measurement of growth rate.** Aerobic growth was on agar plates or in broth in tubes rotated on a Roto-Torque heavy-duty rotator (Cole-Parmer, Vernon Hills, Ill.). Anaerobic growth was in sterile anaerobic Balch tubes or on agar plates incubated in the anaerobic chamber. Cultures were incubated at 37°C.

**Growth rates** were monitored by measuring a change in optical density at 415 nm in sterile 96-well plates (Costar; Corning Inc. Life Sciences, Acton, Mass.) with an automated microplate reader (Bio-Tek instruments, Winooski, Vt.) run with Deltasoft 3 software (BioMetallics, Inc., Princeton, N.J.). For the 96-well-plate experiments, overnight cultures of *E. coli* were diluted 104 in MOPS diluent, and a 10-μl inoculum was inoculated into 200 μl of medium in triplicate.

**RESULTS**

**Sensitivity of *E. coli* BW13711 to WTE under aerobic conditions.** An overnight culture was serially diluted in MOPS diluent and plated on MOPS and 0.1% WTE medium to determine if *E. coli* BW13711 was sensitive to tannins in the WTE. The colony count on MOPS medium was 1 × 108 CFU/ml, but no growth was obtained on 0.1% WTE plates at higher than a 10−3 dilution. At a 10−4 dilution, clusters of colonies grew on 0.1% WTE plates. Colonies were picked from the edge of the clusters and streaked on 0.1% WTE medium. Only three of the seven colonies picked grew, and when these three were transferred onto fresh WTE-containing medium, only one, designated WTT1, produced isolated colonies on 0.1% WTE medium. The other two grew only on densely streaked areas of the plate. The clustering of colonies and density-dependent growth suggested that *E. coli* cells were scavenging...
a diffusible inhibitor, which they could inactivate when cells were present in large numbers.

The MIC of WTE under aerobic conditions was determined for BW13711 and WTT1 in liquid culture (Table 2). Due to density-dependent growth in the presence of tannins, the overnight cultures were diluted 10⁻⁴ before the addition of a 0.05% inoculum to the media. At a concentration of 0.075% WTE, BW13711 grew at a slower rate, and there was no growth on 0.1% WTE medium. However, WTT1 could still grow in 0.1% WTE medium, but no growth was detected in 0.15% WTE medium. WTT1 could therefore tolerate a higher level of WTE in liquid medium but was still susceptible to inhibition when WTE was present above 0.1%.

An experiment was performed to determine if the effect of WTE on E. coli BW13711 was bacterioidal or bacteriostatic under aerobic conditions. An overnight culture was diluted and inoculated into MOPS diluent, 0.1 and 0.2% WTE diluent, and 0.1 and 0.2% WTE medium, and the cell viability was monitored at intervals over a 24-h period. In MOPS diluent, the number of cells increased slightly, indicating that there was some nutrient carryover from the original culture (Table 3). In both diluent and medium containing 0.1% WTE, the number of viable bacteria remained steady for the first 8 h but increased slightly after 24 h, indicating possible detoxification over time (Table 3). The effect of 0.1% WTE in MOPS medium on E. coli BW13711 did not result in a decrease in the number of cells. However, at 0.2% wattle tannin, there were less than 10 CFU/ml after 4 h, indicating that the effect of wattle tannin in the medium is bacterioidal.

Since the initial experiments were carried out with a crude WTE, it was necessary to determine whether the toxicity was due to condensed tannins or other components of the extract. The WTE (65.6% phenols as tannic acid equivalents) was fractionated into three parts, with a 67% extraction efficiency of the tannins. The ethanol fraction contained 60.2% phenols (43% of the original phenolic material), the ethyl acetate fraction contained 102.4% phenols (34%), and the aqueous extract contained 91% phenols (23%). Condensed tannins were present in all three extracts as determined with the vanillin-HCl method on thin-layer chromatography plates. Sensitivity to the various fractions was determined by monitoring growth rates of E. coli BW13711 and WTT1 in medium containing 0.1% of each fraction. Both BW13711 and WTT1 could grow in medium containing the ethanol fraction, but neither grew on the ethyl acetate or aqueous extract-containing media, indicating that sensitivity is due to the tannins present in the WTE.

Condensed tannins oxidize in the presence of oxygen (27), so an experiment was conducted to determine whether auto-oxidation of tannin influences growth of E. coli BW13711. Growth rates were determined with fresh medium and stored refrigerated medium, which was oxidized as judged by a color change. E. coli BW13711 could grow at normal growth rates in oxidized tannin-containing medium but did not grow in fresh tannin-containing medium, suggesting that E. coli BW13711 is inhibited by an unstable auto-oxidation product produced in the medium.

Sensitivity of E. coli BW13711 to wattle tannin in anaerobic conditions. To determine if E. coli BW13711 and WTT1 were sensitive to tannins under anaerobic conditions, a plate count experiment was performed. Dilutions of overnight cultures of BW13711 and WTT1 were plated on MOPS and 0.1% WTE medium. One set of media was made up aerobically and incubated aerobically. Another set was made up aerobically and incubated anaerobically. The third set was made up anaerobically, and cysteine-sulfide solution was added to the medium as a reducing agent. The fourth set was anaerobic medium without cysteine-sulfide solution to exclude any possible effect that cysteine or sulfide may have on the tannin. Growth of E. coli BW13711 under aerobic and anaerobic conditions was inhibited when WTE media was prepared aerobically; however, anaerobic growth of E. coli BW13711 was not inhibited in WTE medium prepared under anaerobic conditions (Table 4). This confirmed that it is a product of tannin auto-oxidation which inhibits E. coli BW13711. The tolerance of WTT1 to these auto-oxidation products is dependent on a gene expressed during growth under aerobic conditions, as WTT1 did not grow on media prepared aerobically but incubated anaerobically (Table 4).

Oxidative stress caused by auto-oxidation of wattle tannins. Oxidation of neighboring hydroxyl groups on the flavonol B ring could result in production of hydrogen peroxide (27). We

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**TABLE 2. Growth rates of E. coli strains on WTE medium with differing concentrations of WTE under aerobic conditions**

<table>
<thead>
<tr>
<th>WTE concn (%)</th>
<th>BW13711 (min⁻¹)</th>
<th>WTT1 (min⁻¹)</th>
<th>TA4131 (min⁻¹)</th>
<th>TA4110 (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.011 ± 0.0007</td>
<td>0.010 ± 0.0005</td>
<td>0.016 ± 0.0014</td>
<td>0.014 ± 0.0007</td>
</tr>
<tr>
<td>0.05</td>
<td>0.009 ± 0.0002</td>
<td>0.009 ± 0.0003</td>
<td>0.013 ± 0.0001</td>
<td>0.011 ± 0.0006</td>
</tr>
<tr>
<td>0.075</td>
<td>0.008 ± 0.0002</td>
<td>0.008 ± 0.0003</td>
<td>0.013 ± 0.0004</td>
<td>0.010 ± 0.0002</td>
</tr>
<tr>
<td>0.1</td>
<td>NG</td>
<td>0.007 ± 0.0001</td>
<td>NG</td>
<td>0.010 ± 0.0003</td>
</tr>
<tr>
<td>0.15</td>
<td>NG</td>
<td>0.009 ± 0.0006</td>
<td>NG</td>
<td>0.009 ± 0.0006</td>
</tr>
</tbody>
</table>

* Strain WTT1 is wattle tannin tolerant; strain TA4110 is a constitutive oxyR mutant. Results are means ± standard deviations (n = 3). NG, no growth after 48 h of incubation at 37°C.

**TABLE 3. Counts of viable E. coli cells after incubation at 37°C under aerobic conditions to determine bacteriocidal or bacteriostatic effect of condensed tannins**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>MOPS diluent</th>
<th>0.1% WTE diluent</th>
<th>0.1% WTE medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.6 ± 0.08</td>
<td>3.6 ± 0.23</td>
<td>3.9 ± 0.09</td>
</tr>
<tr>
<td>4</td>
<td>4.2 ± 0.22</td>
<td>3.6 ± 0.41</td>
<td>3.3 ± 0.19</td>
</tr>
<tr>
<td>8</td>
<td>4.9 ± 0.16</td>
<td>3.1 ± 0.35</td>
<td>3.2 ± 0.07</td>
</tr>
<tr>
<td>24</td>
<td>5.4 ± 0.06</td>
<td>4.7 ± 0.54</td>
<td>5.3 ± 0.22</td>
</tr>
</tbody>
</table>

* Results are means ± standard deviations (n = 3).
hypothesized that hydrogen peroxide is being produced during auto-oxidation of wattle tannins and that this is responsible for the inhibitory effect on *E. coli* under aerobic conditions. Hydrogen peroxide was detected in 0.1% WTE medium; the rate of production, measured over 45 min after addition of the WTE, was determined to be 0.033 μM H₂O₂/min (standard deviation, 0.006). The rate of production in MOPS medium without the addition of tannins was 0.005 μM H₂O₂/min. Adding diethylenetriamine pentaacetic acid, an ion chelator, to the medium resulted in a decreased accumulation rate of H₂O₂ (0.021 μM H₂O₂/min; standard deviation, 0.003). This indicated that metal ions enhanced, but were not essential for, H₂O₂ production. Auto-oxidation of wattle tannins in aqueous conditions therefore resulted in H₂O₂ production, which was higher in the presence of metal ions. We therefore hypothesized that H₂O₂ inhibits growth of *E. coli* BW13711 in WTE medium under aerobic conditions. The addition of 1% catalase (2% wt/vol) (Boehringer Mannheim) to the dilution series of an overnight culture of *E. coli* BW13711 before plating on 0.1% WTE medium resulted in colony counts of 9.1 ± 0.18 log₁₀ CFU/ml compared to 9.2 ± 0.04 log₁₀ CFU/ml on MOPS medium. The colony count on 0.1% WTE medium without catalase was <6 log₁₀ CFU/ml. This experiment indicates that H₂O₂ was inhibiting growth.

To determine whether WTT1 could tolerate higher levels of H₂O₂ than the parent strain, MOPS medium containing various concentrations of H₂O₂ was inoculated. After 48 h, WTT1 had grown on both MOPS medium alone and medium containing 20 and 40 mM H₂O₂. Two of three wells inoculated with BW13711 were turbid in medium containing 20 mM H₂O₂, but no growth was observed at 40 mM H₂O₂. Peroxidase activity of cells in MOPS medium harvested at log phase was 0.004 U/mg of protein (standard deviation, 0.004) for BW13711 and 0.055 U/mg (standard deviation, 0.011) for WTT1. Constitutive peroxidase activity was more than 10-fold higher in the tannin-tolerant mutant, *E. coli* WTT1.

A constitutive OxyR mutant strain TA4110 was shown to tolerate higher levels of WTE medium than either its isogenic parent strain TA4131 or WTT1 (Table 2), again demonstrating that an increase in the oxidative stress response allows *E. coli* strains to overcome the inhibitory effect of wattle tannins under aerobic conditions. An experiment to confirm the importance of HPI in tannin tolerance was done by knocking out the katG gene in *E. coli* BW13711 and WTT1. The katG17::Tn10 null allele was transduced from JI361, and the sensitivity of the resulting transductants (XS01 and XS02) to WTE was determined. The transductants did not grow in liquid medium containing 0.05% WTE (results not shown), indicating that HPI catalase is essential for growth in the presence of wattle tannin auto-oxidation products.

**Genetic analysis of the tannin-tolerant strain, WTT1.** Experiments were performed to identify the mutation that causes WTT1 to overproduce HPI catalase. Our hypothesis was that either the regulatory gene oxyR or the katG gene itself was mutated. To test this hypothesis, we introduced the wild-type genes into WTT1 to determine if peroxidase activity decreased. WTT1 was transduced with P1 virus which had been grown on strain BW6165 (argE86::Tn10). The argE gene is at 89.4 min, oxyR at 89.5 min, and katG at 89.0 min. According to cotransduction frequencies, oxyR will be cotransduced with the selected argE86::Tn10 allele at an 85% frequency and katG will be cotransduced at a frequency of 50%. Eight of 17 (47%) argE::Tn10-transduced colonies screened lost the ability to overproduce peroxidase. This indicated that the mutation was in the katG gene. To ensure that the mutation was not in the oxyR gene, a transductant that contained the argE::Tn10 allele but still overproduced HPI was transduced into strain GS022. GS022 contains wild-type chromosomal oxyR and katG alleles, but it has a lambda bacteriophage bearing a katG::lacZ fusion integrated at the lambda attachment site. Four of 17 (24%) transductants of GS022 overproduced HPI catalase activity by 10-fold. However, these strains did not show any increase in expression of the katG::lacZ fusion, which would have been the case if oxyR had been activated. This indicated that the mutation must be in katG allele itself. To verify this, the promoter region of the katG gene in WTT1 was sequenced, and a C-to-T transversion was detected 1 bp beyond the putative −35 region (Fig. 1).

**DISCUSSION**

Our results indicate that oxidative modification of wattle tannins resulting in hydrogen peroxide production is responsible for *E. coli* BW13711 sensitivity under aerobic conditions. Further, an increase in the oxidative stress response allows *E. coli* strains to overcome the inhibitory effect.

Nonenzymatic auto-oxidation of neighboring hydroxyl groups of the flavonol B ring would result in formation of H₂O₂ (27). These results with complex condensed tannins corroborate results with monomeric polyphenols in which recycling redox reactions between copper(II) and (I) complexed to catechins resulted in bacterial hydrogen peroxide production (11). In our experiments, the effect on *E. coli* BW13711 appeared to be bacteriostatic rather than bactericidal at 0.1%
wattle tannins, but the \( \text{H}_2\text{O}_2 \) accumulation rate was lower in our study (0.033 compared to 0.69 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \)/min with 100 \( \mu \text{M} \) (-)-epigallocatechin and 1 \( \mu \text{M} \) Cu(II)]. At higher tannin concentration (0.2% wattle tannins), the effect on \( E. \text{coli} \) BW13711 was bactericidal. In the presence of a chelator, there was reduction in tannin-produced \( \text{H}_2\text{O}_2 \), indicating that metal ions assist the production of \( \text{H}_2\text{O}_2 \) but are not necessary. Fe\(^{2+}\) and Cu\(^{2+}\) have been shown to cause an increase in the degradation rate and an increase in browning during oxidative modification of the flavonoids quercetin and rutin (quercetin 3-O-rhamnosylglucoside) (14). In our experiments, both iron (7.5 \( \mu \text{M} \)) and copper (0.0117 \( \mu \text{M} \)) were present in WTE medium and may have formed complexes with the tannins.

The production of hydrogen peroxide would vary for different polyphenolic compounds. In the experiments performed by Makris and Rossiter (14), oxidative modification with and without metal ions was more pronounced for quercetin than for rutin. In unpublished experiments, we determined that \( E. \text{coli} \) BW13711 was not sensitive to 0.1% quebracho extract, even though the total phenolic content as tannic acid equivalents of the quebracho extract was higher than that of the WTE (88.7 compared to 65.6%). In comparative studies, it was shown that four tannins differ in the proportion of copper precipitated, with quebracho tannin giving the lowest yield of copper precipitation (15). These authors did not determine copper precipitation by wattle tannin. If metal ion precipitation by wattle tannin is greater than that of quebracho tannin, it may explain why \( E. \text{coli} \) BW13711 was not sensitive to 0.1% quebracho extract. The hydrogen peroxide accumulation rate in 0.1% quebracho tannin medium was one-fourth that of the accumulation in 0.1% WTE medium (data not shown).

The oxidative stress response is necessary for \( E. \text{coli} \) to overcome the inhibitory effect of condensed tannins in the medium. Mutants that lacked the HPI gene, an inducible catalase gene, were more sensitive to the effect of tannins. Mutants overexpressing antioxidant enzymes were less sensitive to the effect of tannins. The wattle tannin-tolerant strain, WTT1, isolated in our experiments had a more-than-10-fold increase in HPI catalase activity. A point mutation 1 base beyond the −35 region of the promoter is responsible for the increase in peroxidase activity in WTT1. Overproducing HPI mutants described in the literature were also indicated to have mutations in the \( \text{katG} \) promoter based on cotransduction frequencies (7). It has been shown that transcription activation at OxyR-dependent promoters is dependent on protein-protein contact between OxyR and the RNA polymerase \( \alpha \) (28). The mutation may allow for the polymerase to bind in the absence of OxyR. Another possibility is that OxyR binds inappropriately to the mutant promoter region under reducing conditions and may induce the protein. OxyR has been shown to make four intermediate-strength contacts along the region to which it binds (30). In some genes, OxyR can function as both an activator and a repressor by binding to different contact points in the promoter region, with reduced OxyR having an elongated DNase I footprint. Reduced OxyR does not bind to the \( \text{katG} \) promoter region (30), but the mutation may allow for inappropriate binding of reduced OxyR and activation of the catalase.

Hydrogen peroxide is an important molecule contributing to oxidative damage in cells. In environments in which tannins produce hydrogen peroxide as a product of auto-oxidation, organisms will be inhibited. This may have an effect in soil systems where tannins will be present in decaying plant biomass. Even in gastrointestinal systems, hydrogen peroxide may be produced during mastication of plant material and in the rumen, as studies have measured levels of up to 1,630 nmol of \( \text{O}_2 \)/liter (25). Hydrogen peroxide is generated in beverages such as coffee and tea due to oxidation of polyphenolic components, and it has been suggested to be the main cause of coffee-induced mutagenesis (22). In many microbial species, the OxyR protein induces antioxidant defense genes in response to hydrogen peroxide. Interestingly, even in fully aerobic media, endogenous \( \text{H}_2\text{O}_2 \) formation appears insufficient to activate the OxyR system (5). Thus the OxyR regulon must have evolved to defend bacteria against external \( \text{H}_2\text{O}_2 \) sources, which have not been described. Tannins might be one such external \( \text{H}_2\text{O}_2 \) source.

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