J Gastroenterol 2007; 42:346–351
DOI 10.1007/s00535-007-2012-5

Journal of Gastroenterology



# Association of tannase-producing *Staphylococcus lugdunensis* with colon cancer and characterization of a novel tannase gene

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***Background.*** The relationship between *Streptococcus (St.) bovis* endocarditis and colon cancer is well known. In *St. bovis,* the biotype I strain (formerly, *St. gallolyticus*) produces tannase that degrades tannins. The aim of this study was to investigate the association of tannase-producing bacteria with colon cancer, and to identify the major tannase-producing bacteria and the gene involved. ***Methods.*** Tannase-producing bacteria were isolated in tannic acid-treated selective agar medium from feces and rectal swabs of 357 patients who underwent colon endoscopy from 1999 to 2004. ***Results.*** Tannase-producing bacteria were isolated more frequently from the colon cancer group (24.3%) than from the adenoma or normal groups (14.4%; $P < 0.05$). *S. gallolyticus*, *Staphylococcus (S.) lugdunensis*, *Lactobacillus (L.) plantarum*, *and L. pentosus* were all identified as tannase-producing bacteria. Of these, *S. lugdunensis* was significantly isolated from the advanced-stage cancer group (22.2%; $P < 0.001$) more than from the early-stage cancer (8.6%) or adenoma (4.9%) groups. The gene (*tanA*) for tannase in *S. lugdunensis* was cloned and sequenced. The *tanA* gene was associated with all *S. lugdunensis* but not with other bacteria by Southern blotting and polymerase chain reaction. ***Conclusions.*** Tannase-producing *S. lugdunensis* is associated with advanced-stage colon cancer, and the *tanA* gene is a useful marker for the detection of *S. lugdunensis*.

**Key words:** tannase genes, *Staphylococcus lugdunensis*, colon cancer, *Streptococcus bovis*

## Introduction

Colon cancer is a major neoplastic disease worldwide. Many studies have reported the association of *Streptococcus* (*St.*) *bovis* endocarditis with colon cancer.[1–4] However, this association has not been investigated. *Streptococcus bovis* has been divided by biotype into three types, I, II/1, and II/2, although it has recently been reclassified both genetically and biochemically.[5–8] Osawa and colleagues isolated tannase-producing *St. bovis*, which is biotype I, from feces of the koala, and designated it *St. gallolyticus*.[8–10] They further speculated that tannase-producing *St. bovis* was the same bacterium isolated from feces and blood of patients with colon cancer, and might be associated with colon cancer.[11,12]

Tannins are water-soluble polyphenolic compounds that are widely distributed in plants.[13] Based on their structure, tannins are classified into two groups, hydrolyzable tannins and condensed tannins. They are bacteriostatic and toxic compounds and form a tannin–protein complex that is resistant to degradation by the digestive enzymes of mammals.[14] Tannase (tannin acylhydrolase) degrades not only tannins such as tannic acid but also the tannin–protein complex by hydrolysis of the ester bonds in hydrolyzable tannins to release gallic acid.[15,16] Tannase is produced by many microorganisms, including fungi, yeast, and bacteria species that are occasionally part of the gastrointestinal tract flora of animals and humans.[15,17–19] However, there is little information about the activity of tannase or the pathogenicity of tannase-producing bacteria.

To investigate the association of tannase-producing bacteria with colon cancer, we screened the tannase-producing bacteria from feces and rectal swabs of humans, including patients with colon cancer. Also, the gene for tannase of *Staphylococcus (S.) lugdunensis*, which was commonly isolated from colon cancer patients in this study, was cloned and characterized.

Received: April 15, 2006 / Accepted: January 18, 2007
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## Materials and methods

### *Patients*

Feces or a rectal swab was used to obtain a sample from 357 patients who underwent colon endoscopy from 1999 to 2004. Patients were divided into a cancer group (107 patients, 61 men and 46 women; mean age, 66.8 ± 11.5 years), an adenoma group (142 patients, 97 men and 45 women; mean age, 65.7 ± 9.2 years), and a normal group (108 patients, 59 men and 49 women; mean age, 64.2 ± 11.5 years) according to endoscopic and pathological findings after samples were taken. The cancer group comprised patients with early-stage cancer (35 patients, 24 men and 11 women; mean age, 64.8 ± 8.5) and advanced-stage cancer (72 patients, 37 men and 35 women; mean age, 67.8 ± 11.0). Informed consent was obtained from all patients.

### *Isolation and identification of tannase-positive bacteria*

A feces sample (approximately 0.2 g), a swab of feces, or a rectal swab was transferred to a tube containing 3 ml of brain heart infusion (BHI; Oxoid, Basingstoke, UK) and *Streptococcus* selective supplement, which included colistin sulfate and oxolinic acid (SSS; Oxoid). The tube contents were mixed using a vortex mixer and incubated anaerobically at 35°C for 24 h. Approximately 0.2 ml of the fecal culture suspension was spread onto tannic acid-treated BHI agar containing SSS (T-BHIS agar) and incubated anaerobically for 4 days at 35°C.[9] After incubation, a clear zone-forming colony was judged to be a tannase-positive bacterium. The purified isolate from the colony underwent Gram-straining, and was identified by biochemical tests with API Staph, API 20Strep, API 40Strep, or API 50CHL (bioMerieux, Montalieu, Vercieu, France). Some bacteria identified by biochemical testing were confirmed by comparison of 16S rRNA sequences.[20,21] *Streptococcus bovis* ACM3611 (formerly, *St. gallolyticus*) was isolated from koala feces by Osawa et al.,[10] and *S. lugdunensis* ATCC43809 was used as type strain for bacterial identification.[22]

### *DNA manipulation*

DNA manipulation was performed as described previously.[23] Plasmid pUC18 was used as a cloning vector. The chromosome DNA of tannase-producing *S. lugdunensis* TP415, which was isolated from the feces of patients with colon cancer in this study, was partially digested with *Hin*dIII. The digested DNA was inserted into the *Hin*dIII site of pUC18, and the ligated DNA was used to transform *Escherichia (E.) coli* DH5α with selection in LB agar containing ampicillin and Xgal. The resulting white colony was transferred onto T-BHI agar, and the cells were incubated anaerobically at 35°C for 4 days; a clear zone-forming colony resulted. The DNA sequence was determined by the primer-walking method using an Applied Biosystems 310 DNA sequencer with a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

### *Southern blotting and polymerase chain reaction*

DNA extraction and Southern hybridization were carried out as described previously.[23–25] Lyses of bacterial cells were carried out with lysozyme, lysostaphin, or achromopeptidase (Wako Pure Chemical Industries, Osaka, Japan). The purified genome DNA was digested completely with *Hin*dIII. The digested DNA was electrophoresed on a 0.7% agarose gel. A 767-bp fragment, which was amplified by polymerase chain reaction (PCR) with the primer set tanA-F-F6 (5′-CGTGGT GCCCTATTACAAGT) and tanA-F-R8 (5′-GCA CAAGCATGTCAACAAGA), was used for the *tanA* probe for Southern hybridization. The probe was labeled with an AlkPhos Direct labeling kit (Amersham Biosciences, Buckinghamshire, England). The template DNA for PCR was prepared as described previously.[26] The PCR assay for the *tanA* gene was performed with the above set of primers with an initial denaturation step of 3 min at 95°C. The first five cycles were at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; the second 25 cycles were at 94°C for 20 s, 53°C for 20 s, and 72°C for 20 s. There was a final extension step at 72°C for 5 min. The PCR products were analyzed by agarose electrophoresis.

### *Nucleotide sequence accession number*

The nucleotide sequence reported here has been deposited in the DDBJ, EMBL, and GenBank databases under accession number AB244239.

### *Statistical analysis*

Statistical analysis was done using the $\chi$-squared and *t* tests. Differences with $P$ values $<0.05$ were considered statistically significant.

## Results

### *Isolation of tannase-producing bacteria from patients with colon cancer*

The patients were divided three groups: a colon cancer group, an adenoma group, and a normal group. Patients with ulcerative colitis were excluded from this experi-

ment. Table 1 shows the number of patients from whom a tannase-producing bacterium was isolated and the species of bacteria identified.

No statistically significant difference in the isolation of tannase-producing bacteria was found between the colon cancer and adenoma groups and the normal group ($P > 0.05$). However, tannase-producing bacteria were isolated more frequently from the cancer group than from the noncancer groups (the adenoma and normal groups) ($P < 0.05$)

Four bacterial species were identified as tannase-producing bacteria and made a clear zone on T-BHIS agar. *S. lugdunensis*, *St. gallolyticus* (formerly, *St. bovis* biotype I), and *Lactobacillus (L.) plantarum* were isolated from the cancer group. *S. gallolyticus*, which is well known to be associated with colon cancer, was isolated from only one patient with colon cancer. *L. plantarum*, which was the most common bacterium across patients, was common in the normal group and *L. pentosus* was isolated from one of the patients in the normal group. However, *S. lugdunensis* was the most frequently isolated bacterium from patients with colon cancer. The difference in isolation frequency of *S. lugdunensis* between the colon cancer group and the normal group was statistically significant ($P < 0.001$). We thus studied further the relationship between *S. lugdunensis* and colon cancer. The colon cancer patients were histologically divided into early-stage and advanced-stage cancer groups. *S. lugdunensis* was significantly more frequently isolated from the advanced-stage cancer group than from the adenoma or normal groups ($P < 0.001$), but no statistically significant difference in frequency was found between the early-stage and the advanced-stage cancer groups ($P > 0.05$).

### *Cloning and characterization of a gene for tannase in* S. lugdunensis

To identify the gene for tannase in *S. lugdunensis*, gene cloning was carried out, using *E. coli* as the cloning host. Approximately 3000 white colonies resistant to ampicillin were tested for the production of tannase using T-BHI agar. Three transformants that formed a clear zone on T-BHI agar owing to the production of tannase were isolated: Tan18, Tan24, and Tan37. Although the three transformants carried plasmids of different sizes, a 7-kb *Hin*dIII fragment was found in each. The nucleotide sequence of the 7-kb *Hin*dIII fragment in pTZtan18 was determined (accession number AB244239); the fragment was 6994 bp and contained three open-reading frames (ORFs). ORF1, ORF2, and ORF3 were theoretically capable of encoding polypeptides of, respectively, 635 amino acids (aa) [molecular weight (MW), 69190], 479 aa (MW, 54987) and 613 aa (MW, 67971) (Fig. 1). When we searched for

**Table 1.** Comparison of isolation of tannase-producing bacteria between colon cancer, adenoma, and normal groups

| Tannase-producing bacteria | No. (%) of patients with tannase-producing bacteria[a] | | | | | | *P* value[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cancer | | | | | | | | | | |
| | Total [357] | All [107] | Early [35] | Advanced [72] | Adenoma [142] | Normal [108] | C vs. N | C vs. (A+N) | (C+A) vs. N | Ec vs. N | Ac vs. N |
| All | 64 (18.0) | 26 (24.3) | 6 (17.6) | 20 (28.2) | 22 (15.6) | 16 (14.9) | 0.078 | 0.044 | 0.306 | 0.281 | 0.090 |
| *Staphylococcus lugdunensis* | 30 (8.4) | 19 (17.8) | 3 (8.6) | 16 (22.2) | 7 (4.9) | 4 (3.7) | <0.001 | <0.001 | 0.023 | 0.108 | <0.001 |
| *Streptococcus gallolyticus* | 1 (0.3) | 1 (0.9) | 0 (0.0) | 1 (1.4) | 0 (0) | 0 (0) | — | — | — | — | — |
| *Lactobacillus plantarum* | 32 (9.0) | 6 (5.6) | 3 (8.6) | 3 (4.2) | 15 (10.6) | 11 (10.2) | 0.141 | 0.1052 | 0.429 | 0.663 | 0.085 |
| *Lactobacillus pentosus* | 1 (0.3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (0.9) | — | — | — | — | — |

[a] Total number of patients diagnosed by colon fiberscope are shows in brackets
[b] Differences with $P < 0.05$ were considered statistically significant
Early, early-stage cancer; Advanced, advanced-stage cancer; Ec, early-stage cancer; Ac, advanced-stage cancer; C, cancer group; N, normal group; A, adenoma group; —, not studied

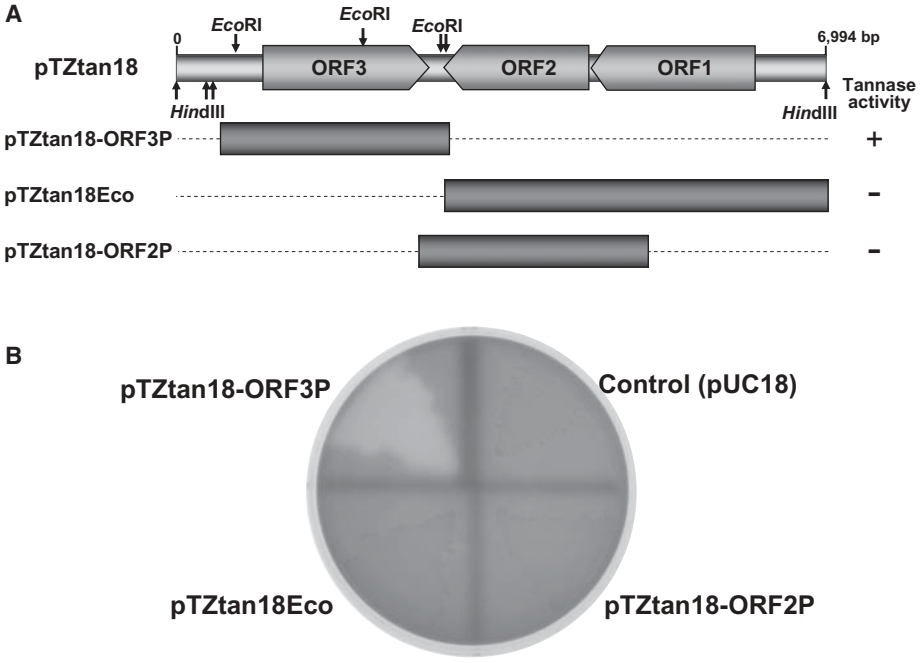


**Fig. 1. A** Physical map of the 7.0-kb *Hin*dIII fragment, including *tanA* and properties of deletion derivatives constructed in vitro. *Arrows* indicate location and orientation of the open-reading frames (*ORFs*). *Bars* indicate DNA region of deletion derivatives and subclones. Tannase is produced (+) or not produced (–) by *Escherichia coli*. **B** Growth of *E. coli* carrying plasmid on T-BHI agar. Tannase activity was judged by the formation of a clear zone around *E. coli* grown on T-BHI agar

similarities in the amino acid sequences using the BLAST and FAST programs, ORF1 and ORF2 proved to be similar to a subunit of the β-glucoside-specific IIABC component of *Listeria monocytogenes* (identity, 37%)[27] and 6-phospho-β-glucosidase of *S. aureus* (identity, 76%).[28] No amino acid sequence predicted from ORF3 exhibited significant homology to those of published genes, including tannase.[29,30] To identify the gene for tannase, we constructed deletion derivatives of pTZtan18 and subcloned ORF2 and ORF3; production of tannase in the strain carrying the plasmid was tested (Fig. 1). The strain carrying ORF3 made a clear zone on T-BHIS agar, although no tannase was detected from the strain carrying ORF1 and ORF2. It follows that ORF3 corresponds to the tannase-producing gene. Thus, ORF3 was designated *tanA*. Our similarity search also showed that *tanA* is a novel gene.

### *Southern hybridization and PCR screening*

We isolated at least four species of bacteria that produce tannase from human feces and rectal swabs. To study the distribution of the *tanA* gene, Southern hybridization was carried out using several bacteria used in the laboratory (Fig. 2). Although *S. lugdunensis* ATCC43809, which is the type strain of *S. lugdunensis*,[22] neither grew nor made a clear zone on T-BHIS agar, the hybridized band was found from all tested *S. lugdunensis* strains, including ATCC43809. No hybridized band was detected in the other bacteria strains tested.

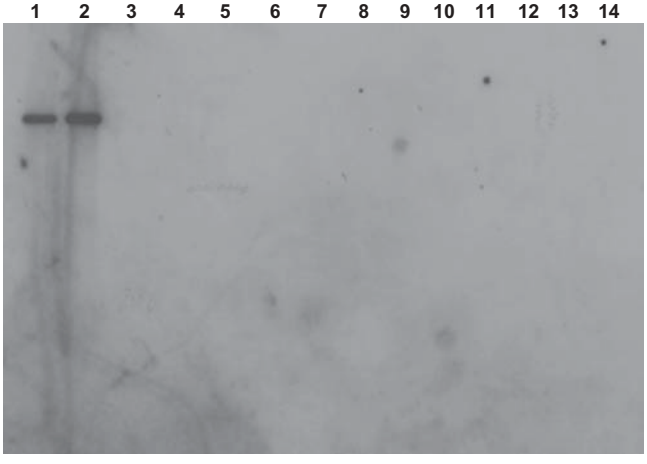


**Fig. 2.** Southern blot hybridization of *Hin*dIII digests of genomic DNA with a probe tanA of *Staphylococcus lugdunensis*. *Lane 1*, *S. lugdunensis* ATCC43809; *lane 2*, *S. lugdunensis* TP457 (positive control); *lane 3*, *S. aureus* ATCC25923; *lane 4*, *S. epidermidis* ATCC14990; *lane 5*, *S. hominis* JCM2419, *lane 6*, *S. schleiferi* ATCC43808; *lane 7*, *Escherichia coli* ATCC11775; *lane 8*, *Bacillus subtilis* ATCC6633; *lane 9*, *Enterococcus faecalis* ATCC19433; *lane 10*, *Lactobacillus plantarum* TOA1008 (isolate from silo); *lane 11*, *L. plantarum* 67 (clinical isolate); *lane 12*, *L. pentosus* St067 (clinical isolate), *lane 13*, *Streptococcus bovis* 303; *lane 14*, *St. gallolyticus* ACM3611. Lanes 2, 11, 12, and 13 were clinically isolated in this study

PCR screening for *tanA* was similarly performed using the clinical strains of *S. lugdunensis* isolated in this study. A PCR product corresponding to *tanA* was detected from all *S. lugdunensis* strains tested, but not from the other bacteria.

## Discussion

We investigated the relationship between tannase-producing bacteria and colon cancer. No significant differences in patient age between the three groups or in sex between the cancer and normal groups were found in this study. In the adenoma group, men were predominant over women. Four bacteria species were isolated from human feces and rectal swabs. *Lactobacillus plantarum* and *L. pentosus* were isolated mainly from the normal group. Isolation of *L. plantarum* and *L. pentosus* has been reported from the feces of normal healthy subjects,[19] and *Lactobacillus* spp. have been used as probiotics for the prevention of colon cancer. Furthermore, although the genome sequence of *L. plantarum* was completed, no gene for the tannase-like protein was found by similarity searches of the amino acid and DNA sequences of *S. lugdunensis* tannase. The clear zone of *Lactobacillus* isolates on T-BHIS agar was obviously different from that of *S. lugdunensis* (data not shown). The results suggest that the biological character of tannase derived from *Lactobacillus* differs from that of *S. lugdunensis* and that the colonization of *Lactobacillus* spp. may be unrelated to colon cancer.

*S. lugdunensis* was isolated from four normal and seven adenoma patients, and a single patient with a previous history of colon cancer was found in the adenoma group. Furthermore, no observation of endocarditis, other intestinal diseases such as ileus, or extraintestinal manifestation was found in the patients from whom *S. lugdunensis* was isolated from feces or swabs of feces. The isolation frequencies of *S. lugdunensis* from the early-stage cancer, adenoma, and normal groups were significantly lower than that of *S. lugdunensis* from the advanced-stage cancer group. This result suggests that tannase-producing *S. lugdunensis* may colonize more easily patients with advanced-stage colon cancer than patients with adenoma or early-stage cancer. Hirota et al.[11] has reported that clinical isolates of *St. gallolyticus*, which produces tannase, express sialyl Lewis$^x$-like oligosaccharides, which are found on the surface of human colon cancer cells.[11] Furthermore, Herrero et al.[3] recently reported that *St. bovis* biotype I (latterly *St. gallolyticus*) was associated with colon cancer, based on a reevaluation of *St. bovis* endocarditis cases.[31] Those results suggest that tannase-producing *S. lugdunensis* may express sialyl Lewis$^x$-like oligosaccharides and be more adhesive to advanced-stage colon cancer cells than to adenoma or normal cells. Further studies are necessary to demonstrate the expression of sialyl Lewis$^x$-like oligosaccharides in *S. lugdunensis.*

We attempted in this study to isolate tannase-producing *St. gallolyticus*, which is frequently isolated from the blood of patients with colon cancer in Western countries. However, *St. gallolyticus* was found in the feces of only one patient with colon cancer. In the present study, since tannase-producing bacteria were selected using T-BHIS agar, tannase-producing *S. lugdunensis* was frequently isolated from the advanced-stage cancer group. Consequently, if T-BHIS agar is used, tannase-producing *S. lugdunensis* could be isolated from the feces of Western people with colon cancer, as well as from Japanese. We believe that this is also the first report that *S. lugdunensis* produces tannase.

*S. lugdunensis*, which is a coagulase-negative *Staphylococcus*, is responsible for serious infections, including infective endocarditis, skin infections, peritonitis, meningitis, and brain abscess.[32–34] Infections of *S. lugdunensis* resemble infection by *S. aureus* rather than by coagulase-negative *Staphylococcus*. Consequently, the development of a simple and rapid means for detecting *S. lugdunensis* appears to have contributed not only to the early finding and prevention of colon cancer but also to the diagnosis of *S. lugdunensis* infection.

For this purpose, cloning and sequencing of *tanA* of *S. lugdunensis* were carried out. A similarity search, Southern blotting, and PCR of *tanA* revealed that *tanA* is a novel gene peculiar to *S. lugdunensis*. It is therefore possible that *tanA* is a useful marker gene for the simple and rapid detection of *S. lugdunensis*, although screening for *tanA* in *S. lugdunensis* strains isolated from other infections such as endocarditis is needed. Cloning of genes for tannase and characterization of tannase in *St. gallolyticus* and *L. plantarum* are in progress.

In summary, this study suggests the possibility that the tannase-producing *S. lugdunensis* is associated with advanced-stage colon cancer. Cloning and characterization of *tanA* shows its value as a detection marker for *S. lugdunensis*.

Clinical tracing of noncancer patients with *S. lugdunensis* is now needed to elucidate the relation between *S. lugdunensis* colonization and colon cancer.

*Acknowledgments*. We wish to thank K. Hojo, A. Tomizawa, T. Ro, R. Takahashi, C. Miwa, K. Ishii, and T. Arai for their technical assistance. We also gratefully thank Dr. R. Osawa for providing us the *St. gallolyticus* ACM3611. This work was supported by the High-Tech Research Centre Project for Private Universities provided by the Ministry of Education, Culture, Sports, Science and Technology, and by the Matching Fund Subsidy for Private Schools of Japan.

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