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Cariogenic Actinomyces Identified with a β-Glucosidase-Dependent Green Color Reaction to *Gardenia jasminoides* Extract

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The oral bacteria *Actinomyces naeslundii* and *Actinomyces viscosus* are known to contribute to the initiation and progression of human dental caries, especially root caries. We report that both *A. naeslundii* and *A. viscosus* react with a component in the *Gardenia jasminoides* extract to produce a distinct green product. This green color reaction was found to be dependent on the bacterial β-glucosidase. The reaction is specific for cariogenic actinomyces, and it can detect as few as 10^4 cells of *A. naeslundii* and *A. viscosus* per ml.

Actinomyces naeslundii and *Actinomyces viscosus* are associated with dental caries (13, 25, 27). Either species alone induces periodontal diseases and dental caries in experimental animals (15). In particular, these two bacteria have been implicated as causes of human root surface caries (4, 6, 16, 24, 25). Although *A. naeslundii* and *A. viscosus* could be distinguished by their fibril types or catalase activities, genetic analysis of human isolates showed that these two species have similar DNAs (5). More importantly, these two actinomyces are almost identical in all other physiological traits (12, 14), and both of them are cariogenic.

Previous studies have suggested a possible association between oral *A. naeslundii* and *A. viscosus* and dental caries (4, 24, 25). Thus, simple and reliable methods for the enumeration of these cariogenic actinomyces will be useful tools for caries diagnosis and risk assessment. Selective medium (9, 29) and antibodies (10, 21, 26) have been used to detect cariogenic actinomyces. Although each of these techniques has its unique positive features, they have their limitations as well. For example, the selective media used for cariogenic actinomyces are only partially selective, and antibody-based methods are relatively complex. The study described here introduces a new, color-based enumeration method for the detection of cariogenic actinomyces.

In the process of a large-scale screening for bioactive compounds in medicinal herbs, we observed that the crude extract of *Gardenia jasminoides* was able to induce green color development in cultures of *A. naeslundii*. In this experiment, the *G. jasminoides* extract was prepared by a water extraction method followed by ethanol precipitation (19) and was stocked at a final concentration of 1 g of dried herb/ml of water. The experiment was performed by the following procedures. First, *A. naeslundii* strain ATCC 12104 was grown at 37°C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) in brain heart infusion (BHI) broth medium (Becton Dickinson, Sparks, Md.). Twenty microliters of each of the overnight cultures (optical density at 600 nm [OD_{600}], 0.6 to 0.8) was inoculated into 2 ml of BHI broth medium containing various dilutions of the *G. jasminoides* extracts and was allowed to grow and develop a green color reaction at 37°C. The minimum concentration of *G. jasminoides* extract required for the green color reaction was 0.5 mg of dried herb/ml. Most experiments described below were performed with 10 mg of dried herb/ml. The green color product has a maximum absorption at 605 nm. The green color first appeared at 10 h (OD_{605}, ≈0.1), and it could easily be recognized by visual examination within 24 h (OD_{605}, >0.15).

To test whether this green color reaction was specific for *A. naeslundii*, we used the same experimental conditions to test a group of other oral bacteria, including *Actinobacillus actinomycetemcomitans* ATCC 33384, *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas gingivalis* ATCC 33277, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguis* ATCC 10556, and *Streptococcus sobrinus* ATCC 6715, as well as actinomyces, including *Actinomyces bovis* ATCC 13683, *Actinomyces denticolens* ATCC 43322, *Actinomyces gerencseriae* ATCC 23860, *Actinomyces israelii* ATCC 12102, *Actinomyces meyeri* ATCC 35568, *Actinomyces odontolyticus* ATCC 17929, and *Actinomyces viscosus* ATCC 19246. The results showed that the green color reaction was specific for cariogenic *A. naeslundii* and *A. viscosus* strains among the bacterial strains tested.

To test the sensitivity of this green color reaction, *A. naeslundii* at initial cell concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 cells/ml was inoculated into BHI medium containing 10 mg of dried herb/ml and examined for a green color reaction at different time points (Table 1). As shown in Table 1, the minimum cell density required for *A. naeslundii* to produce a distinct green color within 48 h is 10^4 cells/ml (Table 1). Similar results were observed when *A. viscosus* was tested (data not show). It is of interest that previous studies suggested that the cell density of *A. naeslundii* and *A. viscosus* in saliva is between 10^5 and 10^7 cells/ml (4, 22), which falls within the detectable range of the green color test.

To characterize the active component in *A. naeslundii* for the color reaction, 10^8 cells in 1 ml of BHI medium was treated by boiling, sonication, or boiling plus sonication. The *G. jasminoides* extract was then added to the treated cultures. The mixtures, including a positive control consisting of untreated cells, were incubated at 37°C. Six hours later, a distinct green

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TABLE 1. Cell density of *A. naeslundii* and the color reaction

Initial cell density (cells/ml)	Color development at the following incubation times (h)[a]:					
	0	2	6	10	24	48
10^8	−	+	++	++	++	++
10^7	−	−	+	++	++	++
10^6	−	−	−	+	++	++
10^5	−	−	−	−	+	++
10^4	−	−	−	−	−	++
10^3	−	−	−	−	−	−

[a] The results of the green color development were defined by −, +, and ++, in which − indicates an OD_{605} <0.05, + indicates a faint green color (OD_{605}, >0.05), and ++ indicates that the color could clearly be detected by visual examination (OD_{605}, >0.15).

color was observed in the positive control, as well as in the sonicated mixture. However, no green color was observed for the heat-treated mixtures even after 48 h. This result implies that living cells are not required to perform the green color reaction and that the active component produced by *A. naeslundii* is heat labile. Since most of the proteins are insensitive to sonication but are easily denatured by heating, it is possible that the active component in *A. naeslundii* is a heat-labile protein.

Escherichia coli strain TOP10 (Invitrogen Corp., Carlsbad, Calif.) was negative for the green color test. Thus, we reasoned that this strain could be used as a host to clone the *A. naeslundii* gene encoding the active protein for the green color reaction. Genomic DNA of *A. naeslundii* ATCC 12104 was purified with the Easy-DNA kit (Invitrogen) and was then subjected to *Bam*HI digestion. The digested DNA fragments were inserted into the *Bam*HI cloning site in the pZErO-2 vector (Invitrogen) and were then transformed into TOP10 cells. Transformants grown on plates with BHI medium, kanamycin (100 μg/ml), and *G. jasminoides* extract were screened for green color development. Three green *E. coli* colonies were identified. Spectrum analysis of the green product produced by these recombinant *E. coli* colonies showed that they had profiles identical to that produced by *A. naeslundii*. The plasmid DNAs were isolated from these colonies, and the DNAs from all three of them contained an identical *A. naeslundii* genomic fragment. One of these plasmids, named pLL1 (Fig. 1), was chosen for further study.

The 5-kb *Bam*HI fragment of *A. naeslundii* genomic DNA in pLL1 was sequenced at the University of California, Los Angeles. Two complete open reading frames (ORFs) were identified. BLAST sequence analyses (1) show that both ORFs share homology with genes involved with the use of β-glucosides. Therefore, these two ORFs were designated *bgl1* and *bgl2*, respectively, in the present study (Fig. 1A). The predicted protein product of *bgl1* has 33% identity to the *Clostridium longisporum* phosphotransferase system enzyme II (PTS II), a protein involved in the transport of β-glucosidic sugars into the cell with concomitant phosphorylation of the sugar (8). Downstream of *bgl1* is *bgl2*, which encodes a predicted protein of 488 amino acids with a calculated molecular mass of 54 kDa (Fig. 2). *The bgl2* open reading frame has 63% identity to the phospho-β-glucosidase of *C. longisporum* (8) and 58% identity to the phospho-β-glucosidase of *Bacillus subtilis* (18) (Fig. 2).

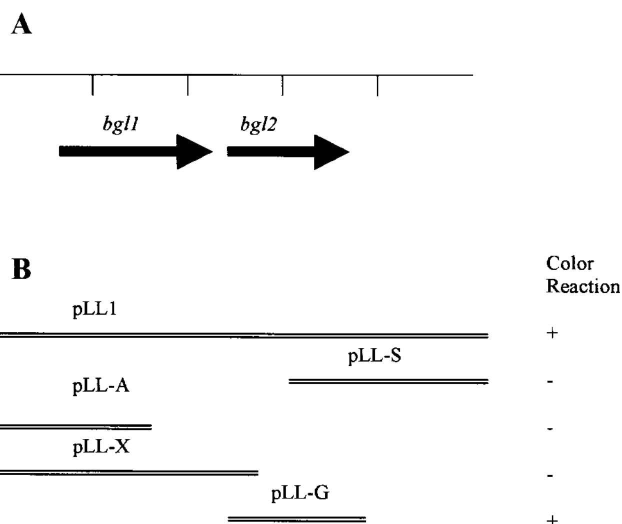

FIG. 1. Map of the cloned *A. naeslundii* insert in pLL1. (A) Identified ORFs. (B) Subclones and their ability to produce the green color reaction in *E. coli*.

Subclones of pLL1 were constructed and subjected to the functional assay to determine which gene was required for the green color reaction. TOP10 cells harboring pLL-X, pLL-S, and pLL-A (Fig. 1B) did not produce the green color, whereas TOP10/pLL-G cells (Fig. 1B) did produce the green color. These results indicate that *bgl2*, but not *bgl1*, is important for the green color reaction.

To verify the predicted β-glucosidase activity of the Bgl2 protein, a group of known glucosides (Table 2) was used to test the *bgl2* gene product for glucosidase activity. Cell lysates made from TOP10/pLL-G served as the source of Bgl2, and TOP10/pLL-X cell lysates were used as a negative control. Briefly, 10^8 cells grown in BHI medium with kanamycin (100 μg/ml) were sonicated and then challenged with various glucosides, each at a final concentration of 4 mM, at 37°C for 30 min. The overall catalytic activity of the mixture against a specific substrate was determined by the released nitrophenol with a maximum absorption at 410 nm (20). The OD_{410} obtained from TOP10/pLL-G was then compared with that obtained from TOP10/pLL-X to determine the activity exhibited by the Bgl2 protein. We found that β-D-glucosides could be efficiently hydrolyzed by the cellular lysates containing the Bgl2 protein, whereas no effect was observed for the lysates containing the BglI protein (Table 2). No significant catalytic activity was observed when β-D-fucoside, β-D-galactoside, or β-D-xyloside was used to challenge the Bgl2 protein (Table 2). Similar results were observed when *A. naeslundii* was assayed (data not show). On the basis of these findings, it is likely that Bgl2 is a β-glucosidase and that the green color reaction is dependent upon a functional β-glucosidase.

β-Glucosidase has been found in a variety of organisms and has significant implications in medical and environmental applications (2, 3, 7, 11, 17, 20, 23). In the present study, we cloned a β-glucosidase from cariogenic *A. naeslundii* and demonstrated that its activity is critical for the generation of a distinct green color product from the *G. jasminoides* extract. *G.*

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A  MTATSTTSKS NPN-FPDGFL WGGATAANQI EGAYNEDGKG LSVQDVMPRG
B  ------MS-S NEKRFPEGFL WGGAVAANQV EGAYNEGGKG LSTADVSPNG
C  --------MS FDLSFPKSFL WGGATAANQF EGAYNEDGKG LSIQDIAPKG

A  IMAHPTQAPT PDNLQARGDR PSTTAYAEDI SLFAEMGFKV FRFSIAWSRI
B  IMSPFDESMT SLNLYHNG-I DFYHRYKEDI ALFAEMGFKA FRTSIAWTRI
C  VMGPITEVPT EDNMKLIG-I DFYHRYKEDI KLFAEMGFKT FRLSIAWSRI

A  FPLGDETEPN EEGLAFYDRV LDELEKHGIE PLVTISHYET PLHLARTYDG
B  FPNGDEEEPN EEGLRFYDDL FDELLKHHIE PVVTISHYEM PLGLVKNYGG
C  FPNGDDEIPN EKGLEFYDKV FDELQRYGIE PLVTLSHYET PLNLSKKYNG

A  WTDRRLIGFF ERYARTLFER YGKRVKYWLT FNEINSVLHE PFLSGGVATP
B  WKNRKVIEFY ERYAKTVFKR YQHKVKYWMT FNEINVVLHA PFTGGGLVFE
C  WANRDLIGFY ERYVRTVFTR YKDKVKYWLT FNEINSAIHA PYMSAGIWTD

A  KDRPPEQDLY QAIQNELVAS AAATRIAHET NPDIQVGCMI LADPTYPLTP
B  EGENKLNAMY QAAHHQFVAS ALAVKAGHDI IPDSKIGCMI AATTTYPMTS
C  KSELSKQDLY QAMHHELVAS ALAVKIGHEI NPDFKIGCMI LGIPVYPLTP

A  DPRDVWAAKQ AERANYAFGD LHVRGEYPGY LRRTLRDKGI ELEITEEDRV
B  KPEDVFAAME NERKTLFFSD VQARGAYPGY MKRYLAENNI EIEMAEGDEE
C  HPDDLIEKMR VERESLFFAD VHARGKYPRY MNRLFKENNI EIKWHEDDAE

A  LLREHTVDFV SFSYYMSVCE TVTQSAEAGR G-NLMGGVPN PTLEASEWGW
B  LLKEHTVDYI GFSYYMSMAA STDPEELAKS GGNLLGGVKN PYLKSSEWGW
C  ILSN-VVDFI SFSYYMSSCA TADEEKKKAG AGNLLAGVPN PYLKASEWGW

A  QIDPAGLRTI LNDYWDRWGK PLFIVENGLG AKDVLVDGPN GP-TVEDDYR
B  QIDPKGLRIT LNTLYDRYQK PLFIVENGLG AVDKVEED-- G--TIQDDYR
C  QIDPKGLRLI LNELYDRYEK PLFIVENGLG AVDELVTDEN GNKTVNDDYR

A  IAYMNDHLVQ VAEAIADGVE VLGYTSWGCI DLVSASTAQM SKRYGFIYVD
B  INYLRDHLIE AREAIADGVE LIGYTSWGPI DLVSASTAEM KKRYGFIYVD
C  IKYLNDHLVQ VAEAIEDGVE LMGYTTWGCI DLVSASTAEL KKRYGFIYVD

A  RDDGGNGTLA RYRKKSFGWY RDVIASNGAS LVPPVQEPPR G
B  RDNEGNGTFN RIKKKSFNWY QQVIATNGES L--------- -
C  RHDDGSGTLE RYKKKSFNWY KEVIATNGKS LER------- -
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FIG. 2. Amino acid sequence of *A. naeslundii* Bgl2 (A) and its alignment with β-glucosidase proteins from *B. subtilis* (B) and *C. longisporum* (C). Matched amino acid residues in these proteins are highlighted in boldface type.

jasminoides is a popular medicinal plant with cholagogic and hemostatic functions (28). Further understanding of the green color reaction could be approached by knockout of the *bgl2* gene in *A. naeslundii* and chemical characterization of the chromogenic compound in *G. jasminoides.*

TABLE 2. Enzymatic activity of Bgl2

Compound	A_{410}[a]	
	TOP10/pLL-X	TOP10/pLL-G
p-Nitrophenyl-β-D-glucopyranoside	0.0575	0.7817
o-Nitrophenyl-β-D-glucopyranoside	0.0272	0.4256
p-Nitrophenyl-β-D-galactopyranoside	0.0514	0.1094
o-Nitrophenyl-β-D-galactopyranoside	0.0532	0.0905
p-Nitrophenyl-β-D-fucopyranoside	0.0321	0.0665
p-Nitrophenyl-β-D-xylopyranoside	0.0250	0.0746

[a] The enzymatic activity was determined by measuring the released free nitrophenol in the reaction mixture at 410 nm. Four millimolar of the tested glucoside in BHI medium was used as a blank for each assay. Similar results were observed when the experiment was repeated three times.

One unique feature provided by the method for detection of cariogenic actinomyces reported here is that the cell density was determined by the total enzymatic activity instead of by enumeration of CFU. It thus gets around a common concern when selective media are used to enumerate clustered bacteria, like *A. naeslundii* and *A. viscosus*. The distinct green color detectable by visual examination also provides a simple and easy way to positively identify the cariogenic actinomyces. The diagnostic implication of this assay needs to be further explored.

Nucleotide sequence accession number. The 5-kb *Bam*HI fragment of *A. naeslundii* genomic DNA in pLL1 was registered with GenBank and given accession number AY029505.

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