JOURNAL OF BACTERIOLOGY, Sept. 2009, p. 5563–5565 Vol. 191, No. 17
0021-9193/09/$08.00+0 doi:10.1128/JB.01226-08
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NOTE

Interspecies Signaling between *Veillonella atypica* and *Streptococcus gordonii* Requires the Transcription Factor CcpA▽

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Received 2 September 2008/Accepted 9 June 2009

***Streptococcus gordonii* and *Veillonella atypica*, two early-colonizing members of the dental plaque biofilm, participate in a relationship that results in increased transcription of the *S. gordonii* gene *amyB*, encoding an α-amylase. We show that the transcription factor CcpA is required for this interspecies interaction.**

Human dental plaque is a well-recognized example of a multispecies bacterial community and a site of interspecies cell-cell interactions. These interactions include physical contacts mediated by molecules on the surfaces of bacterial cells, known as coaggregation, as well as metabolic interactions that have an effect on growth and gene expression (8, 9). Two early-colonizing oral bacteria, *Streptococcus gordonii* and *Veillonella atypica*, undergo such interactions. Fermentation of sugar by *S. gordonii* produces lactic acid, a substrate for energy generation by *V. atypica*. During coculture growth of *S. gordonii* and *V. atypica*, a signaling event that results in increased transcription of the *S. gordonii* gene *amyB*, encoding an α-amylase, occurs (4). We have proposed that AmyB functions intracellularly to hydrolyze stored glycogen, resulting in the availability of sugar for *S. gordonii* and, upon fermentation, lactic acid for *V. atypica* (4). Consequently, *V. atypica*-induced expression of the *S. gordonii* amylase may contribute to cross-feeding between these organisms and may be advantageous to one or both species involved in the symbiosis.

We have previously shown that the signaling between *V. atypica* and *S. gordonii* involves a diffusible signal that has not been identified (4). In other systems, signaling between species of oral bacteria includes the use of autoinducer-2 (8, 9, 14). However, we have been unable to detect autoinducer-2 in *V. atypica* culture supernatants (4). Here we begin to investigate the mechanism by which *V. atypica* causes changes in gene expression in *S. gordonii* by examining the role of the *S. gordonii* transcription factor CcpA in *V. atypica*-induced amylase expression.

The *S. gordonii* amylase encoded by *amyB* was initially identified as a gene that is induced by coculture of *S. gordonii* with *V. atypica* (4). To gain an understanding of the mechanism by which *amyB* transcription is regulated by *V. atypica*, we sought to identify factors in *S. gordonii* that control *amyB* expression. The *amyB* promoter region contains a 7-bp inverted repeat matching the catabolite response element consensus (5) at 11 of 14 positions (4). In other systems, the catabolite response element consensus site is bound by a member of the CcpA family of transcriptional regulators (5). The *S. gordonii* CcpA homolog, previously called RegG, has been previously described (1, 2, 15).

Mutagenesis of *S. gordonii* V288 *ccpA*. To see if CcpA also plays a role in the expression of AmyB during symbiosis with *V. atypica*, we constructed a *ccpA* mutant of *S. gordonii* strain V288 using PCR ligation mutagenesis (10). Two segments of *ccpA* (GenBank accession no. NC_009785), corresponding to bases −9 to 483 and 515 to 964 relative to the ATG start codon, were PCR amplified from *S. gordonii* V288 genomic DNA using primers 5′-*ccpA* (5′-GGAAACAATATGAACAC AGACG-3′) and *ccpA*-XhoI (5′-CGCTCGAGTAACACTTG GAAGTTGGTGC-3′) (for the segment from bases −9 to 483) and primers EcoRI-*ccpA* (5′-GGAATTCCTATCACATTCCT TGCTAAG-3′) and 3′-*ccpA* (5′-GAACAACTTCACGTTCT TCC-3′) (for the segment from bases 515 to 964), where underlining indicates the restriction enzyme sites. The kanamycin resistance gene *aphAIII* was PCR amplified from plasmid pDL276 (3) using primers XhoI-*aphAIII* (5′-CGCTCGAGTG TGGTTTCAAAATCGGCTC-3′) and *aphAIII*-EcoRI (5′-GG AATTCCATCTAAATCTAGGTACTAAAAC-3′). The PCR products were digested with EcoRI and XhoI and ligated together. The product of the ligation was used as the template for a PCR with the 5′-most primer, 5′-*ccpA*, and the 3′-most primer, 3′-*ccpA*. The resulting PCR product was purified and transformed into *S. gordonii* V288 (11). The transformation mixture was plated on Todd-Hewitt agar (Becton Dickinson, Sparks, MD) containing 500 μg/ml kanamycin to select for isolates that had undergone double recombination. PCR anal-

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▽ Published ahead of print on 19 June 2009.

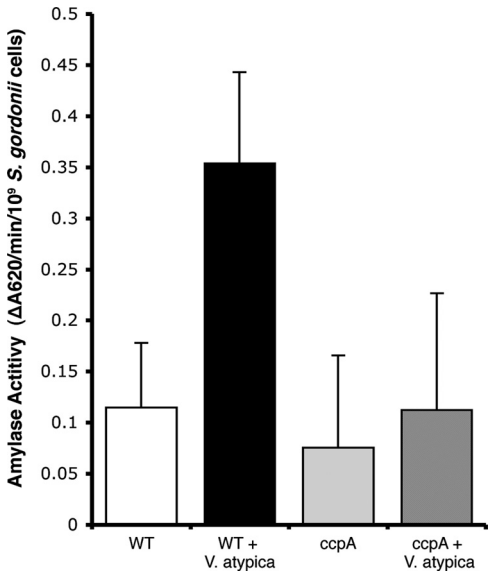

FIG. 1. Amylase activities from wild-type (WT) and *ccpA* mutant *S. gordonii* cells grown alone and in coculture with *V. atypica*. To normalize the activities between monocultures and cocultures, amylase activity is expressed as ΔA_{620}/min/10^9 *S. gordonii* cells. The results presented are the averages from four independent trials. Error bars represent standard deviations.

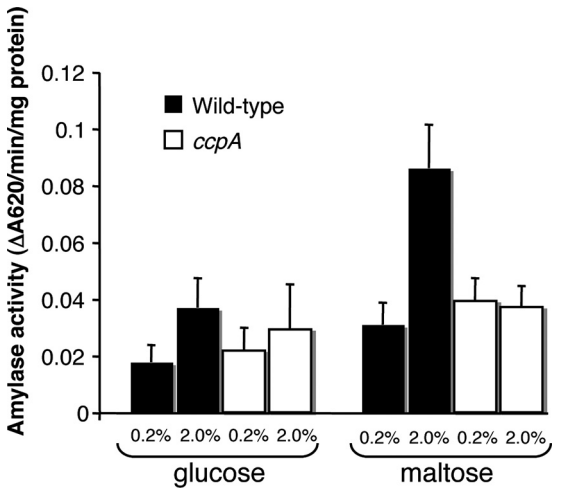

FIG. 2. Amylase activities from wild-type and *ccpA* mutant *S. gordonii* cells grown with either 0.2% or 2% maltose or glucose. The results presented are the averages from four independent trials. Error bars represent standard deviations.

ysis of genomic DNA prepared from one isolate, designated AC001, was performed, and the insertion of the kanamycin resistance gene in *ccpA* was verified.

To verify that insertion of the kanamycin resistance gene in *ccpA* did not result in polar effects on the gene downstream of *ccpA*, reverse transcriptase PCR was performed on the downstream open reading frame (GenBank accession no. NC_009785). RNA was extracted from wild-type and *ccpA* mutant cells grown under the test conditions using the Qiagen RNeasy minikit (Valencia, CA). The RNA was treated with DNase I from New England Biolabs (Ipswich, MA) and used as a template for reverse transcriptase PCR with primers 5′GT (5′-CCTCTTACATGCCGCCAAGAG-3′) and 3′GT (5′-CAATCTGGCATCCTTCTAGCC-3′). DNA fragments of the expected size, 390 bp, were amplified from cDNA of both the wild-type and *ccpA* mutant strains. Controls in which the RNA templates were used for conventional PCR with no reverse transcriptase did not yield PCR products (data not shown).

The growth rates of the wild type and *ccpA* mutant in tryptone-yeast extract medium (TYE; 1% tryptone, 0.5% yeast extract, 0.3% K_2HPO_4) containing either 0.2% or 2.0% glucose or maltose were tested. Under all four conditions tested, the wild type and *ccpA* mutant had doubling times averaging 50 to 60 min, with no statistical difference between results for the two strains.

CcpA is required for *V. atypica*-induced expression of *S. gordonii amyB*. To examine the possibility that CcpA is involved in the ability of *S. gordonii* to regulate *amyB* expression in response to *V. atypica*, we measured the amylase activities of the wild-type and *ccpA* strains grown as monocultures and as cocultures with *V. atypica* PK1885 (7) in medium containing 0.2% glucose. Amylase activity was measured using variations on a previously described method (12). Cell extracts were prepared from 1.0 ml of logarithmic cultures or cocultures. Cells were harvested by centrifugation, washed and resuspended in 50 mM Tris-HCl (pH 6.8), and lysed by bead beating with 0.1-mm glass beads. To compare the amylase activity in cell extracts of *S. gordonii* cells grown in coculture with *V. atypica* to those of cells grown as monocultures, it was necessary to express amylase activity relative to the number of *S. gordonii* cells rather than the amount of protein in cell extracts. Therefore, to normalize the amylase activity to the number of *S. gordonii* cells in the culture, samples were taken prior to harvesting, serially diluted, and plated to determine the number of *S. gordonii* cells per sample. Wild-type *S. gordonii* grown in the presence of *V. atypica* induced amylase activity threefold over the level in *S. gordonii* cells grown alone ($P = 0.007$) (Fig. 1). However, amylase activity in the *ccpA* mutant remained at basal levels and was unaffected by growth in coculture with *V. atypica* (Fig. 1).

Maltose induces CcpA-dependent expression of *amyB*, independently of coculture with *V. atypica*. Characterization of *amyB* homologs from other streptococci has shown induction of enzyme activity by growth in the presence of various carbohydrates (16). The possible role for CcpA in the expression of *amyB* in response to carbohydrates was tested by measuring the amylase activities of both wild-type and *ccpA* mutant *S. gordonii* cell extracts grown in TYE with glucose or maltose. When grown on either 0.2% or 2.0% glucose, the amylase activities of the wild type and mutant were very similar (Fig. 2). In contrast, amylase activity from wild-type *S. gordonii* grown as a monoculture was induced nearly threefold by the growth with 2% maltose (Fig. 2), mirroring the threefold induction by coculture with *V. atypica*. This induction in response to high concentrations of maltose was absent in the *ccpA* mutant. This result suggests that induction of *amyB* by coculture with *V. atypica* and by maltose may involve a common regulatory pathway.

CcpA is required for transcription of *amyB* in coculture biofilms with *V. atypica*. The possibility that CcpA is required for increased transcription from the *amyB* promoter during the growth of *S. gordonii* and *V. atypica* PK1910 (7) in a mixed-

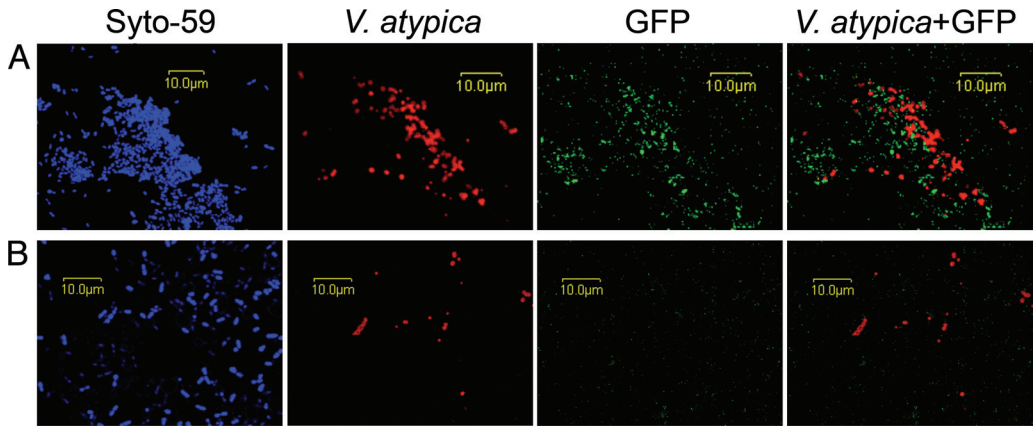

FIG. 3. Confocal scanning laser microscopy analysis of dual-species biofilms of the wild type (A) and *ccpA* mutant (B) with *V. atypica*. The four images in each row are maximum projections of a single field of view showing fluorescence from Syto-59 (blue; all cells), Alexa Fluor 546-conjugated anti-*V. atypica* antibodies (red), and GFP (green; *S. gordonii* expressing *amyB*). The three fluorescence channels are shown separately in the left three panels and, in the right panel, as an overlay of GFP with *V. atypica*.

species biofilm community was investigated using techniques for biofilm growth and imaging described previously (4, 13). A plasmid containing the *amyB* promoter region fused to a promoterless *gfp*, p*Pamy-'gfp* (4), was used to assess transcription from the *amyB* promoter in the wild-type and *ccpA* mutant *S. gordonii* strains. Coculture biofilms of either wild-type or *ccpA* mutant *S. gordonii* containing p*Pamy-'gfp* and *V. atypica* were grown for 4 hours with saliva as the sole nutrient source. Biofilms were stained for all cells present with the DNA stain Syto-59 (Invitrogen, Carlsbad, CA) and for *V. atypica* cells by primary immunofluorescence with Alexa Fluor 546-conjugated anti-*V. atypica* antibodies (6). While wild-type *S. gordonii* cells expressed green fluorescent protein (GFP) when growing in mixed-species microcolonies with *V. atypica*, the *ccpA* mutant did not (Fig. 3A and B). Neither the wild type nor the *ccpA* mutant induced *amyB* expression in the absence of *V. atypica* (not shown). These results further demonstrate that pathways involving CcpA are required for signaling between these organisms in mixed-species biofilms.

In this study, CcpA was found to be required for induction of the *S. gordonii* amylase gene, *amyB*, in response to growth with *V. atypica*. The fact that induction can occur based on the presence of maltose, as well as by coculture with *V. atypica*, suggests that the signal supplied by *V. atypica* could be maltose or a related sugar from the lipopolysaccharide in the *V. atypica* outer membrane. Alternatively, independent signaling pathways may converge, with CcpA responding to an unrelated signal from *V. atypica*. This information provides an understanding of the requirements for the transcription of *amyB* and the signaling pathways that are involved. Further analysis of the activators of CcpA activity, the signal derived from *V. atypica* and protein machinery in *S. gordonii*, will be required to understand the mechanisms for changes in gene expression that result from this symbiosis.

This work was supported by NIH grant 2 P20 RR016479 from the INBRE Program of the National Center for Research Resources.

We thank P. E. Kolenbrander for generously supplying the anti-*V. atypica* antibodies. We acknowledge the Western South Dakota DNA Core Facility (WestCore) at Black Hills State University for sequencing of PCR products. We thank Peg Ustad for her expert assistance with formatting the confocal images. We thank Tomoko Ise for expert technical assistance.

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