Development of Competence for Genetic Transformation of \textit{Streptococcus mutans} in a Chemically Defined Medium

Kunal Desai, a Lauren Mashburn-Warren, a Michael J. Federle, a,b,c and Donald A. Morrison a

Laboratory for Molecular Biology, Department of Biological Sciences, College of Liberal Arts and Sciences, University of Illinois at Chicago, Chicago, Illinois, USA a; Center for Pharmaceutical Biotechnology, University of Illinois at Chicago College of Pharmacy, Chicago, Illinois, USA a; and Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago College of Pharmacy, Chicago, Illinois, USA a

\textit{Streptococcus mutans} develops competence for genetic transformation in response to regulatory circuits that sense at least two peptide pheromones. One peptide, known as CSP, is sensed by a two-component signal transduction system through a membrane receptor, ComD. The other, derived from the primary translation product ComS, is thought to be sensed by an intracellular receptor, ComR, after uptake by oligopeptide permease. To allow study of this process in a medium that does not itself contain peptides, development of competence was examined in the chemically defined medium (CDM) described by van de Rijn and Kessler (Infect. Immun. 27:444, 1980). We confirmed a previous report that in this medium \textit{comS} mutants of strain UA159 respond to a synthetic peptide comprising the seven C-terminal residues of ComS (ComS_{11-17}) by increasing expression of the alternative sigma factor SigX, which in turn allows expression of competence effector genes. This response provided the basis for a bioassay for the ComS pheromone in the 100 to 1,000 nM range. It was further observed that \textit{comS} (but not \textit{comS} mutant) cultures developed a high level of competence in the late log and transition phases of growth in this CDM without the introduction of any synthetic stimulatory peptide. This endogenous competence development was accompanied by extracellular release of one or more signals that complemented a \textit{comS} mutation at levels equivalent to 1 \mu M synthetic ComS_{11-17}.

Over 70 bacterial species are known to have the capacity for natural genetic transformation (10). In some, such as \textit{Neisseria gonorrhoeae}, competence for DNA uptake and incorporation is described as constitutive. In many others, competence in laboratory cultures depends on coordinately regulated expression of gene sets encoding effectors of DNA transport and recombination. Among the streptococci, a number of species develop competence transiently during laboratory culture under conditions that are not completely understood. Central to this regulation in each case, however, is a conserved streptococcal alternative sigma factor, SigX or ComX, that directs the transcription of more than a dozen unlinked operons encoding competence effector proteins (3, 12, 14, 24, 26, 31).

\textit{S. mutans}, a common human commensal associated with dental caries, has been the subject of intensive genetic characterization. Particularly valuable for this work has been the discovery of several strains that are capable of natural genetic transformation, facilitating directed genetic manipulations. In these strains, competence for genetic transformation is not constitutively expressed but develops during growth in certain media and environments in the laboratory. Competence has been variously reported to develop transiently in lag phase (26) or during exponential growth (16, 20, 23, 28, 29). The conditions that favor such development of competence remain poorly characterized, but a common feature of competence protocols for \textit{Streptococcus mutans} has been growth in the rich medium Todd-Hewitt Broth (THB) supplemented with heat-inactivated horse serum. A major advance toward understanding competence development in \textit{S. mutans} was the discovery that a peptide pheromone, called CSP (competence-stimulating peptide), both coordinates the production of several nonlantibiotic bacteriocins known as mutacins and strongly up-regulates SigX and the competence cascade (15, 24). The peptide is processed posttranslationally, both during export (at a consensus GG site of proteolysis) and after export, where removal of three C-terminal residues increases the specific activity of the mature pheromone 10-fold (25). CSP acts through a two-component signal transduction system receptor, ComD, and a cognate response regulator, ComE, which in turn directly stimulates transcription from the promoters of three unlinked mutacin genes (bsmA, bsmB, and bsmC), a related transporter gene (nmlA), and a putative mutacin immunity protein gene (immB) (9, 29). However, the pathway transmitting this signal to stimulate expression of SigX is unclear, beyond the fact that it requires mutacin V (also known as CipB, SMU.1914, NlmC, or BsmA), intact \textit{comS} and \textit{comR} genes, and a functional oligopeptide permease (4, 17, 24).

Mashburn-Warren et al. (17) recently reported that a second peptide pheromone receptor system, known as type II ComR/ComS, is required to link the CSP response to SigX induction and competence in \textit{S. mutans}. Type I ComR proteins, characteristic of the salivarius group of streptococci, recognize a peptide with the sequence PF(A/T)GCL that is produced as a C-terminal fragment of the product of the gene \textit{comS} and that regulate transcription of \textit{comS} and \textit{sigX} (5), while the type II ComR proteins, found in all species of the pyogenic, bovis, and mutans groups of streptococci for which genome sequences are available, are proposed to recognize peptides with a different consensus sequence, XNXDXXXWXX (17). Since a synthetic \textit{S. mutans} ComS peptide (specifically, the seven C-terminal residues of ComS, designated ComS_{11-17}) induces robust expression of SigX and high levels of transformation in the absence of ComE and appears to be at the
core of competence regulation in *S. mutans*, it is of interest to characterize this pheromone system in more detail. The observation that response to ComS11-17 requires the opp oligopeptide permease suggests that a critical step in this pathway is the uptake of a small peptide pheromone signal and suggests that this part of the pathway could be sensitive to competition by the peptides that are abundant in peptone-containing media, such as THB, much as was reported for the type I ComR/ComS system of *Lactococcus lactis* (6). Thus, we were especially interested in the possibility of using a chemically defined medium (CDM) devoid of exogenous peptides for such studies. Indeed, endogenous competence development is reported to be superior in CDM compared to THB with horse serum (27). However, the resulting optimal, but short-lived, competence condition was obtained at a low cell density (the maximum was at an optical density at 550 nm [OD550] of ~0.04) during the lag phase of culture growth in anaerobic CDM and allowed transformation of only 1/1,000 or fewer of the cells (27). In contrast, Mashburn-Warren et al. (17) reported that synthetic ComS11-17 can induce sigX expression and development of a high level of competence in exponential CDM cultures and noted that expression of the sigX promoter is low during exponential growth but increases during the transition to stationary phase in such CDM cultures.

To facilitate further studies of the nature and production of the native signal of the type II ComR/ComS system of *S. mutans*, we investigated the development of competence in a chemically defined, peptide-free culture medium and employed bacterial LuxAB reporter fusions to directly monitor expression from the sigX promoter. Here, we report that CDM cultures at low density respond to ComS11-17 by switching to a pervasive and persistent state of competence. We further describe endogenous competence development in CDM in the late exponential or transitional phase of growth and show that it is preceded by elaboration of a secreted signal that complements a comS defect.

**MATERIALS AND METHODS**

**Strains, plasmids, and media.** Strain UA159 was kindly supplied by Lin Tao. Other strains and plasmids are described in Table 1. Liquid cultures were grown in closed screw-cap 13-mm- or 18-mm-diameter glass tubes at 37°C in CDM or THB (Difco). Stocks were stored at −80°C after supplementation with 1/9 volume glycerol. DNA ligase, *Taq* polymerase, and restriction enzymes were obtained from Invitrogen. Synthetic ComS11-17 prepared by custom synthesis was obtained from NeoPeptide (Cambridge, MA) and was stored at −20°C in dimethyl sulfoxide (DMSO). Peptides specific for *Streptococcus agalactiae* 2603, *Streptococcus porcinus*,

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype (phenotype) or characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH10C</td>
<td><em>E. coli</em> cloning vector</td>
<td>8</td>
</tr>
<tr>
<td>UA159</td>
<td>Transformable <em>S. mutans</em> isolate</td>
<td>28</td>
</tr>
<tr>
<td>MW04</td>
<td>UA159 ΔoppΔIsp (Spc&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>17</td>
</tr>
<tr>
<td>MW07</td>
<td>UA159 ΔcomRΔcomS pWAR300 (Cm&lt;sup&gt;+&lt;/sup&gt; Erm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>17</td>
</tr>
<tr>
<td>MW17</td>
<td>UA159 ΔcomRΔcomS pWAR304 (Spc&lt;sup&gt;+&lt;/sup&gt; Erm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>17</td>
</tr>
<tr>
<td>MW30</td>
<td>UA159 pWAR312 (Erm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWAR304</td>
<td>pFED761 derivative carrying P&lt;sub&gt;s&lt;/sub&gt;gX-luxAB between the Sall and NotI sites; 6,466 bp (Erm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>17</td>
</tr>
<tr>
<td>p7INT</td>
<td>Shuttle-suicide vector that integrates at the streptococcal bacteriophage T12 attB site</td>
<td>18</td>
</tr>
<tr>
<td>pWAR312</td>
<td>Integrative derivative of p7INT carrying P&lt;sub&gt;s&lt;/sub&gt;gX-luxAB between the XbaI and BamHI sites; 7,129 bp (Erm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**FIG 1** Design of reporter plasmids used to monitor expression of competence regulons. (A) Elements of the type II ComR cell-to-cell communication circuit proposed as the proximal regulator of sigX expression. ComR is proposed to be a direct regulator of the transcription of comS and sigX. ComS acts as a cell-cell signal, but the steps of its processing into a mature form (XIP) and its export are unknown. SigX is the proximal regulator of late competence effector genes. (Reproduced, with permission, from part of a figure in reference 17.) (B) Organization of the replicative plasmid pWAR304, a luxAB transcriptional reporter of expression from the sigX promoter. *ori*, replication origin from plasmid pWVO1 from *Lactococcus lactis* (11); P<sub>s</sub>gX, fragment upstream of sigX; RepA, repA gene from pWW01. (C) Organization of the integrative plasmid pWAR312, a luxAB transcriptional reporter of expression from the sigX promoter. *attP*, integrase target; *attB*, insertion site in the genome of UA159; *int*, integrase gene; *ermB*, rRNA methyl transferase from pAMβ1; *ori*, replication origin from plasmid pUC18 (18).
and Streptococcus parauberis (MGWWNMG, KDWWHIG, and NDWW YIG, respectively (17)) were obtained from the same source. CDM (30) was prepared from concentrated stock solutions as described by Chang et al. (1), sterile filtered, and stored at 4°C. For selection, samples were embedded in THB containing 0.75% agar on a 1.5% THB agar base and covered with a layer of 1.5% THB agar, followed by THB agar containing selective antibiotics. Each agar layer was 3 ml (for 60-mm-diameter plates) or 10 ml (for 100-mm-diameter plates). The plates were incubated at 37°C for 30 to 40 h in 5% CO₂ or in a candle jar. Selective levels of antibiotics were 1.5 µg erythromycin/ml, 200 µg spectinomycin/ml, 7.5 µg chloramphenicol/ml, or 200 µg kanamycin sulfate/ml.

Construction of MW30. The pWAR312 reporter plasmid (Fig. 1) was assembled by amplification of p<sub><i>gac</i></sub>-lux<sub><i>AB</i></sub> from pWAR304 using primers LMW26/LMW27 (LMW26, TCTAGATCGACTGAGCTGTTAGC ATA; LMW27, GGATCCGCGATATCGAAAAATATACATGTT), followed by insertion into p7INT using the XbaI and BamHI sites (in boldface) and cloning in BH10C. To generate the integrated p<sub><i>gac</i></sub>-lux<sub><i>AB</i></sub> reporter strain MW30, pWAR312 was transferred into <i>S. mutans</i> UA159 as described previously (17).

Transformation assay. Unless otherwise indicated, the cultures monitored for development of competence were initiated by 1:50 dilution of a stock prepared by freezing a CDM culture at an OD₅₅₀ of 0.5. Transformation was carried out by adding donor DNA to 0.5-ml culture samples and incubating them for a fixed time at 37°C in Eppendorf tubes before selective plating. When appropriate, DNA exposure was terminated by adding pancreatic DNase I (Sigma Chemical Co., St. Louis, MO) to 7 µg/ml.

Bioassay of pheromone/activator. Indicator cells of strain MW17 were frozen with 10% glycerol after growth to an OD₅₅₀ of 0.5 in CDM. For assay of the activator, cells were thawed at 0°C and used within 2 h. Each assay reaction mixture contained 200 µl cells and 200 µl CDM containing synthetic ComS₁₁₋₁₇ or culture filtrate. After incubation at 37°C in open 500-µl Eppendorf tubes for 50 min, samples of 50 to 100 µl were transferred to wells of white 96-well plates (BD 353296; Becton Dickinson, Franklin Lakes, NJ), exposed to vapor from decanal spread on a plate lid for 2 min, and promptly examined for luminescence in a scintillation counter. ComS₁₁₋₁₇ equivalents in culture filtrates were estimated by comparison of serial dilutions with standard curves determined using serial dilutions of synthetic ComS₁₁₋₁₇.

Donor DNA was prepared by purification of genomic DNA from a lysate of cells that had been treated with 3% glycerol in THB (60 min; 37°C), collected by centrifugation at 4°C, washed in cold distilled water, treated with 0.1% lysozyme in 1/50 volume Tris (10 mM, pH 8)-EDTA (1 mM) buffer containing 25% glucose (60 min; 37°C), and held for 20 min at 55°C after addition of sodium dodecyl sulfate to 1%.

RESULTS

Synthetic ComS₁₁₋₁₇ induces development of a persistent highly competent state in low-density CDM cultures of UA159. To begin to characterize the development of competence in CDM further, the effect of synthetic ComS₁₁₋₁₇ on low-density cultures was examined in more detail than previously reported by varying critical parameters of the experiment. Following introduction of ComS₁₁₋₁₇ in a concentration of 0.1, 1, or 10 µM, expression of a Lux<sub><i>AB</i></sub> reporter linked to the sig<sub><i>X</i></sub> promoter increased dramatically, up to 300-fold above the signal for untreated controls. The luminescence signal continued to increase linearly for up to 4 h in all three cases. After a lag of about 30 to 60 min, competence for transformation began to increase dramatically, as well (Fig. 2). As measured by production of transformants following brief exposures to donor DNA, the resulting competent state persisted for approximately 2 h, after which the rate of transformation dropped to about 20% of its maximum but remained well above the background in untreated controls. This pattern of persistent competence contrasts with the typical streptococcal transient expression of competence; in <i>S. pneumoniae</i>, for example, competence induced by CSP peaks by 20 min and disappears altogether by 50 min (7). However, it is consistent with the temporal patterns reported previously for <i>S. mutans</i> (16, 20). Synthetic ComS₁₁₋₁₇ also had a dramatic physiological effect, reducing the growth rate significantly in a dose-dependent manner (Fig. 2 and 3A), indicating a pervasive response to ComS₁₁₋₁₇ throughout the culture. These responses to ComS₁₁₋₁₇ did not reflect a nonspecific effect of small peptides or small peptides encompassing adjacent tryptophanyl residues, as three similar double-tryptophan heptapeptides failed to stimulate competence or sig<sub><i>X</i></sub> expression or to have any depressive effect on growth (Fig. 2). Maximal competence was achieved using synthetic ComS₁₁₋₁₇ at a level of 1 µM or higher (Fig. 3B). The yield of transformants depended linearly on the DNA concentration, reaching a half-maximal yield above 10⁻⁷/ml at ~5 µg of genomic DNA per ml (Fig. 3C). To ask whether com-
Endogenous competence development is accompanied by elaboration of an extracellular activator. Endogenous development of competence in this CDM offered an opportunity to directly test the idea, proposed earlier (17), that a ComS-derived peptide signal, designated XIP (sigX-inducing peptide), is secreted during competence development by using the new bioassay to determine the level and timing of accumulation of XIP or another pheromone in CDM cultures of MW30 growing to high density. As shown in Fig. 5, filtrates prepared from cultures at densities above an OD$_{550}$ of 0.4 did indeed exhibit P$_{sigX}$-stimulating activity in such an assay. Parallel controls confirmed the absence of such activator activity in cultures of the comS mutant MW17, whereas the WT UA159 behaved like MW30 in producing similar levels of activator at high cell densities (data not shown). From the temporal pattern of these events, we conclude that as CDM cultures of...
UA159 approach stationary phase, endogenous development of competence follows two preliminary phases in which sigX and comS promoters are activated and high levels of one or more derivatives of ComS accumulate in the extracellular fluid.

DISCUSSION

S. mutans has been recognized as a naturally transformable species for over 30 years, and it has been understood since the earliest studies that its competence is a regulated trait. However, the mechanism by which competence is controlled in the species remains unclear. The standard method for achieving competence in the laboratory is culture in THB with added horse serum, where competence develops transiently at low cell densities (16, 20, 23, 27–29). The active components of the medium that promote development of competence, if any, are unknown, and while a variety of bacterial proteins have been reported to modulate such competence development, few of their specific relevant targets have been identified. Intercellular signaling is thought to play an important role in competence regulation, and two chromosomally encoded peptides that have strong and specific stimulatory effects are already known. One, CSP, controls the expression of several of the bacteriocins known as mutacins via a two-component signal transduction system, ComDE; the other, XIP, is thought to mediate a type II ComR/ComS autocatalytic signaling loop. Both CSP and a more active C-terminally shortened derivative of CSP have been recovered from culture filtrates after CSP stimulation (25), but the native XIP molecule has not yet been characterized chemically. Indeed, neither of these peptide pheromones has yet been isolated from cultures developing competence without stimulation by synthetic peptides.

Members of a new family of Rgg proteins, designated type II ComR regulators, were recently proposed to act as part of a quorum-sensing circuit that includes ComR-regulated promoters that direct expression of the comS and sigX genes in a pattern conserved throughout the pyogenic, bovis, and mutans groups of streptococci (17). In the case of S. mutans, both comR and comS are required for competence development in response to the CSP

FIG 4 Bioassay of ComS11-17. (A) Kinetics of response of the comS mutant to ComS11-17. Samples (250 μl) of MW17 (ΔcomS PsigX-luxAB) growing in CDM at an OD550 of 0.4 were mixed with 250-μl volumes of CDM containing ComS11-17 at twice the indicated final concentrations. During incubation at 37°C, 60-μl portions were removed at the indicated times for exposure to decanal and determination of luminescence. (B) ComS11-17 bioassay standard curve. The titration curve was constructed using the MW17 luminescence values at 80 min from the experiment in panel A.

FIG 5 Endogenous competence development in CDM. (A) Development of competence, expression from PsigX, and pheromone secretion by strain MW30 (PsigX-luxAB) during growth in CDM in parallel closed culture tubes. The tubes were opened on reaching the indicated OD550 values, and their contents were distributed for three assays. MW07 donor DNA (0.5 μg) was added to one 500-μl sample from each culture; after 15 min at 37°C, DNase was added and dilutions were plated for Com' transformants after 60 min of further incubation. Triplicate 150-μl samples from each tube were exposed to decanal vapor in a 96-well plate for 2 min before determining luminescence. A 4-ml filtrate of the remainder (0.2-μm filters) was prepared for determination of XIP as comS-complementing activity (ComS11-17 equivalents) by bioassay, as described in Materials and Methods. (B) Growth kinetics of cultures assayed for panel A.
pheromone, and a synthetic ComS derivative both stimulates competence and complements a comS defect. It was hypothesized that endogenous competence development depends on a secreted type II ComS-derived signal, termed XIP, but no direct evidence for production of this signal was available. The present data support that hypothesis by showing the production of a comS-dependent secreted signal that complements a comS mutation and that appears in CDM cultures just before development of competence for DNA uptake. These results set the stage for isolating and identifying the native signal by providing conditions for endogenous development of competence in a culture medium that itself is devoid of extraneous peptides (see the accompanying article by Khan et al. [10a]). It is interesting that maximal expression of sigX was achieved somewhat before XIP activity reached its maximum. We interpret this as a reflection of the autocatalytic nature of the ComS/ComR circuit and conclude that an understanding of the details of initiation of the expression of this circuit may require more sensitive assay tools than were used here.

The present results, showing endogenous development of competence in CDM as cultures approach stationary phase, suggest that in S. mutans, in contrast to streptococci of the mitis, anginosus, and salivarius groups, competence may be a characteristic of certain high-density culture conditions. This interpretation is consistent with the report by Li et al. (15) that cells in S. mutans biofilms either are themselves highly competent or rapidly become so when exposed to fresh medium and donor DNA. The more recent discovery that competence development is stimulated strongly by the hdpR/M system, which itself is activated at very high cell densities (19, 21, 22), is also consistent with this view.

Competence for genetic transformation is expressed transiently in multiple well-studied streptococci, including species of the mitis, anginosus, and salivarius groups. In S. pneumoniae, for example, competence development can be coordinated so that all cells of a culture suddenly and simultaneously upregulate the competence regulon and then downregulate expression of the same genes, creating a temporary but pervasive state of competence lasting little more than 15 to 20 min (2). An unidentified product of the pneumococcal competence regulon itself appears responsible for this rapid negative feedback (13), independent of the continued high levels of CSP. Endogenous competence development by S. mutans in THB has also been commonly described as a transient state, appearing during early or mid-log phase. Development of competence in S. mutans in CDM as response to synthetic ComS11–17 contrasts with all these behaviors, as competence persists for hours, with a reduced growth rate that is reminiscent of the competent state of Bacillus subtilis. Indeed, the present results could be interpreted as indicating that in S. mutans competence represents a stable alternative mode of growth. It is possible that this state is also characteristic of the competent subpopulation expressing sigX after CSP stimulation in rich media, as described by Lemme et al. (14).

The apparent reduction in the growth rate that we found to be one aspect of the response to ComS11–17 in CDM is reminiscent of the response to CSP in rich media, where an apparently lower growth rate is accompanied by the death of a significant fraction of the population (14, 24). In that case, the cell death has been attributed to the CSP-induced bacteriocin CipB when it overcomes immunity provided by Cipl. It is therefore interesting that activation of competence by ComR/ComS, thought to act downstream of CipB, produces a similar effect, suggesting that some of the slowed growth occasioned by CSP may be an indirect effect of the CipB pathway.

ACKNOWLEDGMENTS

L.M.-W. is a Howard Hughes Medical Institute Fellow of the Life Sciences Research Foundation. Some of this work was supported by grants NIH AI091779 from the NIAID (to M.J.F.) and MCB1020863 from the NSF (to D.A.M.).

We thank Indranil Biswas for plasmid pHG91:ISS1, Kevin McVver for plasmid pLZ12Spe, and Alketa Zyka for assistance with many of the experiments reported here. We are grateful to Lin Tao for gifts of strains and for helpful technical advice.

REFERENCES


