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# Deletion of competence-induced genes over-expressed in biofilms caused transformation deficiencies in *Streptococcus mutans*

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## SUMMARY

**Previous studies identified nine genes with increased expression in *Streptococcus mutans* biofilms of which six possessed putative ComX promoter sequences and were homologous to competence-induced genes in *Streptococcus pneumoniae*, *Streptococcus gordonii* and *Bacillus subtilis*. As competence increases in biofilms, a study was undertaken into the roles that these biofilm-induced genes might play in transformation. Only five of the nine gene deletions had a significant effect on transformation efficiency. Deletion of the genes for recombinase A, *recA*, DNA processing protein, *dprA* and single-stranded DNA-binding protein, *ssbA*, produced results comparable with those from other bacteria, supporting the contention that these proteins have similar functions in *S. mutans* competence. The uncharacterized genes *SMU.769* and *SMU.836* produced results in variance to deletion mutants of putative homologues in *S. pneumoniae*. Deletion of *SMU.769* reduced chromosomal transformation 2.3-fold. *SMU.769* belongs to a family of conserved genes induced by the competence-stimulating peptide and which have no established function. In contrast, deletion of *SMU.836* reduced transformation of both plasmid and chromosomal DNA to <3%. Homology searches suggested that Smu.836 belongs to a family of competence-induced peptidoglycan hydrolases with a conserved enzyme domain and a species-variable cell-binding domain for which the best characterized member is the choline-binding protein D, CbpD, of *S. pneumoniae*.**

## INTRODUCTION

The development of natural competence requires the expression of specific proteins involved in the uptake, protection and ultimate integration of donor DNA. In *Streptococcus mutans* the development of competence is regulated by a quorum sensing system, which responds to threshold levels of a peptide pheromone, the competence-stimulating peptide (CSP) (Li *et al.*, 2001, 2002). In *S. mutans* the competence quorum sensing system controls the expression of an alternative sigma factor, ComX, which in other streptococci is known to induce the expression of the majority of the genes required for competence (Rimini *et al.*, 2000; Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004; Vickerman *et al.*, 2007).

DNA transformation during competence involves the binding, uptake and protection of donor DNA followed either by integration into the host chromosome or re-circularization of a plasmid replicon. In Gram-positive bacteria, the process of transformation has been best characterized in two model systems, *Streptococcus pneumoniae* and *Bacillus subtilis*

(reviewed in Lunsford, 1998; Cvitkovitch, 2001; Chen & Dubnau, 2004; Johnsborg *et al.*, 2007; Johnsborg & Håvarstein, 2009). In these bacteria, donor double-stranded DNA is bound to the cell and then transported through the peptidoglycan cell wall by a competence pseudopilus structure (Chen & Dubnau, 2004; Johnsborg *et al.*, 2007). Transport through the membrane, via a pore, involves the degradation of one strand of the DNA resulting in the entry of only single-stranded (ss) DNA (Mejean & Claverys, 1993; Chen & Dubnau, 2004; Johnsborg *et al.*, 2007). This ssDNA is immediately bound by a DNA-processing protein, DprA, which protects the DNA from degradation (Berge *et al.*, 2002, 2003). DprA promotes the binding of the recombinase A protein, RecA, to the DNA (Mortier-Barriere *et al.*, 2007). RecA initiates homologous strand exchange with chromosomal DNA, a reaction which is promoted by the competence ssDNA-binding protein (SSB), possibly by sequestering the excess strand to inhibit the re-exchange of DNA similar to the promotion of *in vitro* strand exchange reactions by housekeeping SSBs (Steffen & Bryant, 2000; Steffen *et al.*, 2002; Grove *et al.*, 2005; Morrison *et al.*, 2007; Mortier-Barriere *et al.*, 2007). In contrast, plasmid replicons can be reconstructed from ssDNA fragments in a process that does not require, but is promoted by, RecA (Lorenz & Wackernagel, 1994). Although much of the mechanics of DNA processing has been deduced from studies observing the fate of donor DNA, and the roles of some key competence proteins have been studied, there remains the potential for other proteins to be involved in the development of competence and the processing of donor DNA.

In *S. mutans*, the level of competence is higher in biofilms than in associated planktonic cultures (Li *et al.*, 2001) and previous proteomic studies have identified nine proteins with increased expression in biofilms (Rathsam *et al.*, 2005a, b). The putative ComX promoter sequence, known as a *cin*-box (also known as the com-box), was identified upstream of six of these genes: *recA*, *dprA*, *ssbA*, *ssuRB*, *SMU.769* and *SMU.836* (Rathsam *et al.*, 2005b). Homologues of these genes are found in the alternative sigma factor regulons (so called 'late genes') of *S. pneumoniae and Streptococcus gordonii* or, in the case of *B. subtilis*, are directly controlled by the master competence regulator, ComK (Rimini *et al.*, 2000; Berka *et al.*, 2002; Hamoen *et al.*, 2002; Ogura *et al.*, 2002; Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004; Vickerman *et al.*, 2007; see Supporting Information Table S1). Recently, a microarray analysis of genes regulated by the *S. mutans* competence quorum sensing system confirmed that these genes were induced by the presence of synthetic CSP (sCSP) (Perry *et al.*, 2009). Of these nine biofilm-induced proteins only RecA has a confirmed role in *S. mutans* competence (Quivey & Faustoferri, 1992). In the current study, deletion mutants were created either to confirm or to investigate the potential role of these genes in transformation.

## METHODS

### Chemicals and enzymes

Unless otherwise stated chemicals and enzymes were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) or were of the purest grade available.

### Bacterial strains and growth conditions

*Streptococcus mutans* LT11 (Tao *et al.*, 1993) and derivatives (Table 1) were maintained in brain–heart infusion broth with or without 0.3% yeast extract (BHIY or BHI; Oxoid Australia, Adelaide, SA, Australia). All batch cultures were grown at 37°C with 5% $CO_2$. For growth rate measurements, overnight cultures were diluted 1 : 80 in fresh BHI and grown anaerobically at 37°C. Doubling times were calculated during exponential growth between optical densities at 600 nm ($OD_{600}$) of 0.100 and 0.600. Where antibiotic selection was required for *S. mutans*, erythromycin, kanamycin or spectinomycin was used at a concentration of 15 μg ml$^{-1}$, 750 μg ml$^{-1}$ or 1 mg ml$^{-1}$ respectively, whereas for *Escherichia coli*, ampicillin and spectinomycin were both used at concentrations of 100 μg ml$^{-1}$. Blue/white selection of cloned plasmids made use of 0.5 mM isopropyl-β-D-thiogalactoside and 80 μg ml$^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

### Electron microscopy

One-millilitre cultures, grown to mid-exponential phase ($OD_{600}$ ~ 0.300–0.600) were centrifuged (10,000 ***g***, 10 min, 20°C) and fixed overnight in Karnovsky's fixative and processed and embedded in

**Table 1** Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference and/or source |
|---|---|---|
| *Streptococcus mutans* | | |
| LT11 | Derivative of strain UA159 | (Tao *et al.*, 1993) |
| IDR1smu769 | *SMU.769::Em*$^r$, deletion of the *SMU.769* gene | This study |
| IDR1smu836 | *SMU.836::Em*$^r$, deletion of the *SMU.836* gene | This study |
| IDR1ssbA | *SMU.1967::Em*$^r$, deletion of the *ssbA* gene | This study |
| IDR1dprA | *SMU.1001::Em*$^r$, deletion of the *dprA* gene | This study |
| IDR1recA | *SMU.2085::Em*$^r$, deletion of the *recA* gene | This study |
| IDR1ssuRB | *SMU.506::Em*$^r$, deletion of the *ssuRB* gene | This study |
| IDR1smu209 | *SMU.209::Em*$^r$, deletion of the *SMU.209* gene | This study |
| IDR1smu961 | *SMU.961::Em*$^r$, deletion of the *SMU.961* gene | This study |
| IDR1cysK | *SMU.496::Em*$^r$, deletion of the *cysK* gene | This study |
| IDR1Δ2028Kn | *SMU.2028::Kn*$^r$, deletion of the *ftf* gene | This study |
| *Escherichia coli* | | |
| XL1-Blue | *endA1 gyrA96(nal*$^R$*) thi-1 recA1 relA1 lac glnV44 F'[ ::Tn10 proAB*$^+$ *lacI*$^q$ *Δ(lacZ)M15] hsdR17(*$r_K^-$ $m_K^+$*)* | Stratagene, La Jolla, CA, USA |
| DH5α | *F*$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(*$r_K^-$ $m_K^+$*), λ–* | (Sambrook *et al.*, 1989) |
| Plasmids | | |
| pVA8912 | pVA8911 without 412-bp *Pvu*II fragment, *Em*$^r$ | (Malke *et al.*, 1994) |
| pVA838 | *Em*$^r$, *Cm*$^r$ | (Honeyman *et al.*, 2002) |
| pDL276 | Shuttle vector with blue/white selection, *Kn*$^r$ | (Dunny *et al.*, 1991) |
| pDL278 | Shuttle vector with blue/white selection, *Sp*$^r$ | (LeBlanc *et al.*, 1992) |
| pEK7152 | pVA838, *Em*$^r$, *Kn*$^r$ | Dr Rathsam, IDR |
| pGemTeasy | Blue/white selection, *Ap*$^r$ | Promega |
| pDL278:769 | pLD278 containing the gene and putative promoter of *SMU.769* | This study |
| pDL278:836 | pLD278 containing the gene and putative promoter of *SMU.836* | This study |

TAAB LV resin (TAAB Laboratories Equipment Ltd, Aldermaston, UK) using standard procedures. Ultrathin (75-nm) sections were stained with uranyl acetate and Reynolds' lead citrate and viewed in a Philips CM10 microscope at 80 KV.

## DNA preparation

Plasmid DNA was prepared from *E. coli* strains with a plasmid purification kit (Qiagen, Doncaster, VIC, Australia). Genomic DNA was prepared from *S. mutans* and its derivatives using the method described by Ushiro *et al.* (1991) with modifications. Cells were lysed in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) containing 2.7 mg ml$^{-1}$ lysozyme, 2000 U ml$^{-1}$ mutanolysin, 33 μg ml$^{-1}$ RNAseA and 4 mU ml$^{-1}$ proteinase K (Qiagen) at 56°C for 1–3 h. To complete the lysis, 1% sodium dodecyl sulfate was added and the incubation was continued for a further 20 min at 56°C. Protein was removed with phenol/chloroform extraction using Sigma light-phase divider gels according to the manufacturer's protocol. Genomic DNA was then precipitated in 0.3 M sodium acetate pH 5.2 with 2.5 volumes of ice-cold ethanol (Fronine, Taren Point, NSW, Australia). Plasmid DNA was prepared from *S. mutans* using a combination of the lysis steps used to extract genomic DNA and the plasmid purification kit (Qiagen) to remove protein and genomic DNA.

## Polymerase chain reaction conditions

Primers were designed with the programs Oligo Analyzer and Oligo Explorer (Teemu Kuulasmaa, http://molbiol-tools.ca/molecular_biology_freeware.htm) and were obtained from Sigma Genosys Australia (Castle Hill, NSW, Australia). Polymerase chain reactions (PCR) used Platinum PCR SuperMix containing recombinant *Taq* DNA polymerase (Invitrogen, Mulgrave, VIC, Australia). The PCR products were purified using the UltraClean PCR Clean-up kit (MOBIO Laboratories, Carlsbad, CA, USA).

### Production of naturally competent cells for targeted gene deletion

Batch cultures of *S. mutans* grown in BHIY with 5% heat inactivated horse serum (Amyl Media, Kings Langley, NSW, Australia) became naturally competent in late exponential phase when the $OD_{600}$ reached 0.450–1.000. For transformation, 200 μl of culture was diluted 1 : 2 in freshly prepared BHIY containing 5% heat inactivated horse serum and 10–100 ng of the appropriate DNA was added. In all instances, a control transformation made use of 75 μg pVA838 plasmid DNA (Table 1).

### Deletion of genes using PCR ligation mutagenesis

Target genes were deleted by PCR ligation mutagenesis, using the bipartite ligation method (Lau *et al.*, 2002), replacing the gene with an *Em*$^r$ cassette. Primers A1 and A2 were used to amplify the *Em*$^r$ cassette (see Supporting Information Table S2) from pVA8912 or pVA838 (Table 1), while the upstream (5′) and downstream (3′) flanking regions of each target gene were amplified from the genomic DNA of *S. mutans* using the appropriate target gene primers 1 and 2, and primers 3 and 4, respectively (Table S2).

The PCR ligation mutagenesis was also used to create genomic DNA containing *Kn*$^r$ for chromosomal transformation analysis. The *Kn*$^r$ cassette was amplified using primers K1 and K2 (Table S2) from pDL276 (Table 1). The fructosyltransferase gene, *ftf* (*SMU.2028*), was chosen as the target gene, because this deletion was unlikely to interfere with transformation (Quivey & Faustoferri, 1992; Table S2).

### Confirmation of gene deletions

Deletion of each target gene was confirmed in two ways. In the first method, genomic DNA from each mutant was amplified by PCR targeting the *Em*$^r$ cassette using primers A1 and A2 (Table S2) or the gene of interest using the appropriate gene primers F and R (see Supporting Information Table S3). A successful gene deletion was positive for the erythromycin gene and negative for the target gene. The opposite was the case for the control using wild-type genomic DNA. In the second method, Southern hybridization analysis was used to confirm the insertion site of the antibiotic cassette in each mutant. Internal PCR products of each target gene as well as the *Em*$^r$ cassette were obtained using gene primers F and R (Table S3) and labeled to create probes using the digoxigenin DNA labeling kit according to the manufacturer's guidelines (Roche Diagnostics Australia, Castle Hill, NSW, Australia). Mutant and parental genomic DNA were digested with either *Eco*RI or *Hin*dIII (New England Biolabs, Ipswich, MA, USA) and duplicate Southern blots (Sambrook *et al.*, 1989) were hybridized with either the *Em*$^r$ cassette or the target gene probe. The probe was detected using the anti-digoxigenin Alkaline Phosphatase Fab fragments (Roche Diagnostics Australia) and Alkaline Phosphatase Conjugate Substrate kit (Bio-Rad Laboratories, Gladesville, NSW, Australia) according to the manufacturer's protocols. The band pattern created was compared with *in silico Eco*RI and *Hin*dIII digests of the respective genomic DNA sequences (ApE v1.12, M. Wayne Davis; http://molbiol-tools.ca/molecular_biology_freeware.htm).

### Complementation of mutants

The target gene including its putative ComX promoter, PCR amplified from *S. mutans* genomic DNA using the 'pro' and 'ter' primers listed in Table S2, was cloned into the pGemTeasy vector (Promega, Sydney, NSW, Australia; Table 1) and transformed into the *E. coli* strain DH5α (Table 1) using ampicillin selection. The purified plasmid was sequentially digested with *Hin*dIII and *Bam*HI (New England Biolabs) and the insert was purified using a QIAquick gel extraction kit (Qiagen) following agarose gel electrophoresis. The insert was then cloned into the *Hin*dIII and *Bam*HI sites of the shuttle vector pDL278 (Table 1) and transformed into *E. coli* strain DH5α (Table 1) using spectinomycin selection. The insert was confirmed by sequencing.

Transformation (see below) of the complementing plasmid (or pDL278 as a control) into the corresponding mutant strain was performed in the presence of the 21-amino-acid sCSP, SGSLSTFFRLFNRSFTQ ALGK (Invitrogen), with erythromycin added to prevent transfer of the complementing gene from the plasmid to the chromosome. Transformants were selected using spectinomycin and the resulting strains were maintained in media containing both

erythromycin and spectinomycin to prevent chromosomal insertion and plasmid curing, respectively. To confirm the identity of the resulting strains, plasmids were isolated and sequenced (see above).

### Transformation of *S. mutans* wild-type and mutant strains

Overnight cultures of *S. mutans* were diluted 1 : 30 into fresh BHI, with or without sCSP, at a concentration of 500 ng $ml^{-1}$. After 2 h at 37°C and at an $OD_{600}$ between 0.100 and 0.200, 1 μg $ml^{-1}$ pEK7152 plasmid DNA (Table 1) or 10 μg $ml^{-1}$ IDR1Δ2028Kn chromosomal DNA (Table 1) was added and the incubation was continued for 1 h at 37°C before placing the cells on ice. Serial dilutions of cultures were then plated on BHI agar with or without 750 μg $ml^{-1}$ kanamycin and incubated anaerobically at 37°C for 48 h with 5% $CO_2$. As mutant strains possessed reduced growth rates in the presence of the selective antibiotics, erythromycin and spectinomycin, strains containing complementing plasmids were transformed in the absence of antibiotics as described above. To confirm that the complementing plasmid had not been integrated or lost during the transformation process, serial dilutions of cultures were also plated on BHI agar containing erythromycin and spectinomycin with or without 750 μg $ml^{-1}$ kanamycin. Transformations were performed in triplicate along with control cultures containing no added DNA to detect spontaneous mutations. Transformation efficiency was calculated as the percentage of transformants in the total viable population, which was determined by the number of colony-forming units on antibiotic-free agar plates.

### Homology searches

Results for PSI-BLAST (Altschul *et al.*, 1997; Schaffer *et al.*, 2001), were obtained from the LANL website (http://www.oralgen.lanl.gov/oralgen/bacteria/smut/), using the protein sequences from the *S. mutans* UA159 genome database (Ajdic *et al.*, 2002) to search the non-redundant protein sequences database with the system default parameters (NCBI; http://www.ncbi.nlm.nih.gov/). Domain information was obtained from the NCBI database, the COG database (Tatusov *et al.*, 2001), the Pfam database (Finn *et al.*, 2008) or the CDD database (Marchler-Bauer *et al.*, 2007). Sequence alignment made use of the CLUSTALW algorithm (Thompson *et al.*, 1994).

Throughout the text, genes are designated by the GenBank number in italics (e.g. *SMU.769*; Benson *et al.*, 2008) and where there is no existing nomenclature the corresponding protein is designated by the number in protein format (e.g. Smu.769).

### Lysis assay

Cell lysis was measured at 44°C in 20 mM potassium phosphate buffer pH 6.5 containing 1 M KCl, 10 mM $CaCl_2$ and 1 mM $MgCl_2$, using the method described by Shibata *et al.* (2005).

### Statistical analysis

The SEM was calculated from three independent experiments and the significance (*P*-value) was calculated using Student's *t*-test.

## RESULTS

### Growth rate and morphology

Gene deletion mutants of *cysK* (*SMU.496*), *ssuRB* (*SMU.506*), *dprA* (*SMU.1001*), *ssbA* (*SMU.1967*), *recA* (*SMU.2085*), *SMU.209*, *SMU.769*, *SMU.836* and *SMU.961* were confirmed by PCR and Southern hybridization (data not shown). Only the *recA* mutant possessed a significantly reduced growth rate compared with the parental strain (Table 2). The mutant strains did not show any morphological defects using Gram staining (data not shown). Electron microscopy of the IDR1smu836 and IDR1smu769 strains also failed to show any differences in morphology compared with the parental strain (data not shown).

### Transformation

Natural competence without the addition of sCSP produced low numbers of transformants (<0.01%; see Supporting Information Table S4). The addition of sCSP improved transformation efficiency 100- to 1000-fold (Table S4). However, it should be noted that even with saturating levels of sCSP, cells that became competent represented only a fraction of the total population (<5%).

**Table 2** Growth rates of deletion mutants compared with the parental strain

| Strain | Average doubling time (min ± SEM)[1] | Significance (*P*) |
|---|---|---|
| LT11 | 53.16 ± 1.16 | – |
| IDR1recA | 61.22 ± 1.87 | <0.02 |
| IDR1dprA | 54.64 ± 0.92 | <0.37 |
| IDR1ssbA | 53.94 ± 1.85 | <0.74 |
| IDR1smu769 | 54.84 ± 2.05 | <0.52 |
| IDR1smu836 | 54.37 ± 1.20 | <0.51 |
| IDR1ssuRB | 53.88 ± 1.78 | <0.75 |
| IDR1smu209 | 54.02 ± 1.20 | <0.63 |
| IDR1smu961 | 53.15 ± 1.80 | <1.00 |
| IDR1cysK | 51.95 ± 3.19 | <0.74 |

[1]Doubling times were calculated from the period of exponential growth between $OD_{600}$ 0.100 and 0.600 from three independent experiments.

Only five of the nine gene deletions had a significant effect on transformation efficiency (Fig. 1, Table S4). Deletion of *recA* abolished transformation of chromosomal DNA and reduced the transformation of plasmid DNA almost 100-fold. The mutation of *dprA* virtually eliminated both plasmid and chromosomal transformation, the levels being <0.01% of the wild-type, which were below the detection limit of the assay. Similarly, the deletion of *SMU.836* resulted in a loss of both plasmid and chromosomal transformation (to <3% of wild-type levels). In contrast, the deletion of *ssbA* did not affect plasmid transformation, while chromosomal transformation was reduced 4.3-fold. The deletion of *SMU.769* also only affected chromosomal transformation; the reduction being 2.3-fold. The other gene deletions did not significantly affect the transformation of either plasmid or chromosomal DNA (Fig. 1, Table S4). For all strains there was no evidence of spontaneous mutation in the control transformations without DNA (data not shown).

Plasmids carrying the gene and putative promoter were created to complement the *SMU.836* and *SMU.769* mutants. The *SMU.836* gene deletion was partially complemented by pDL278:836, increasing the transformation efficiency about five-fold, from <3% to 12–15% of the wild-type not carrying the plasmid (Fig. 2, see Supporting Information Table S5). In contrast, the *SMU.769* gene deletion was not complemented by pDL278:769 (Table S5). Colony counts from plates containing erythromycin and spectinomycin confirmed that neither chromosomal erythromycin resistance nor the plasmid-borne spectinomycin resistance had been lost during the transformation assay (data not shown).

## Analysis of Smu.836

To compare the transformation results of the *S. mutans* deletion mutants with previous studies in other species, *in silico* analysis was used to confirm the identity of protein homologues in these other species (Table S1) and to obtain putative functions for the uncharacterized genes/proteins targeted in this

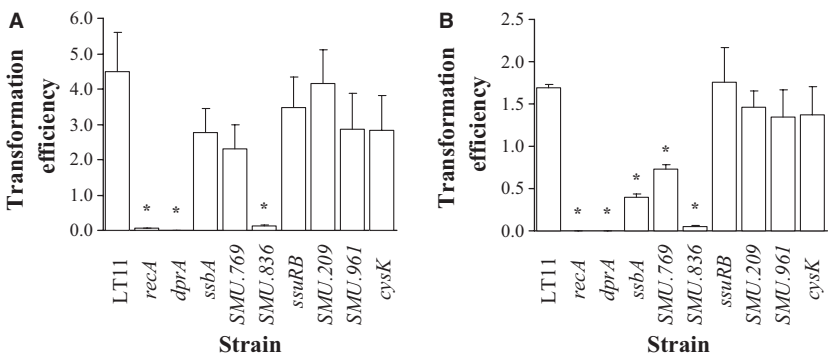


**Figure 1** Effect of gene deletion on synthetic competence-stimulating peptide (sCSP) -induced transformation in *Streptococcus mutans* LT11. Transformation efficiencies of the parental and deletion strains were obtained by exposing batch cultures incubated with 500 ng $ml^{-1}$ sCSP to 1 μg $ml^{-1}$ pEK7152 plasmid DNA (A) or 10 μg $ml^{-1}$ IDR1Δ2028Kn chromosomal DNA (B). Transformation efficiency was calculated as the percentage of transformants in the total viable population using data from three independent experiments. Error bars indicate the SEM. Statistically significant changes ($P < 0.05$) in transformation efficiency are shown (*). The colony counts for plasmid and chromosomal transformations of the *dprA* mutant and the chromosomal transformation of the *recA* mutant were below the statistical limit of detection for the assay.

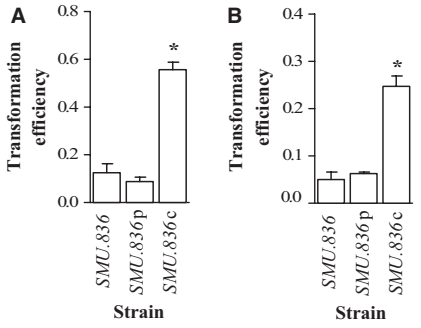


**Figure 2** Complementation of the *SMU.836* gene deletion in *Streptococcus mutans* LT11. Transformation efficiencies of the parental *SMU.836* mutant, the pDL278 vector control (*SMU.836*p) and the pDL278:836 complemented strain (*SMU.836*c) were obtained by exposing batch cultures incubated with 500 ng $ml^{-1}$ sCSP to 1 μg $ml^{-1}$ pEK7152 plasmid DNA (A) or 10 μg $ml^{-1}$ IDR1Δ2028Kn chromosomal DNA (B). Transformation efficiency was calculated as the percentage of transformants in the total viable population using data from three independent experiments. Error bars indicate the SEM. Statistically significant changes ($P < 0.05$) in transformation efficiency are shown (*). Maintenance of both the erythromycin gene deletion and the pDL278 plasmid in the complemented and vector control strains was confirmed by plating on erythromycin/spectinomycin-selective plates.

study, in particular, Smu.836. This analysis suggested that the uncharacterized Smu.836 possessed the characteristics of a peptidoglycan-degrading enzyme with two modular domains. The first of these was a putative cell wall peptidoglycan hydrolase or endopeptidase domain belonging to the NlpC/P60 domain superfamily (CDD clan No: cl11438), which contains both the CHAP (Pfam No: PF05257) and the NlpC/P60 (Pfam No: PF00877) hydolases. Though now designated as an NlpC/P60 domain, the Smu.836 hydrolytic domain showed a higher similarity to the consensus sequence of the CHAP domain than the NlpC/P60 domain (Fig. 3). The second domain was a putative cell-wall-binding domain (CWB) domain. Although the enzyme domain showed a high level of conservation across species, the species variability of the CWB domain limited the matching of Smu.836 to homologues within the *Streptococcus sanguinis* and *S. gordonii* genomes, which encode proteins with similar CWB domains. However, the choline-binding protein D, CbpD, of *S. pneumoniae* was identified as a potential member of this family based on the two parameters of 'late' competence-induction and a protein containing both a CHAP domain and a CWB domain. The CWB domains of the *S. mutans*, *S. sanguinis* and *S. gordonii* proteins contain GBS Bsp-like repeats (Pfam No: PF08481), whereas the CWB domain found in *S. pneumoniae* contains putative CWB repeats (Pfam No: PF01473) and SH3b sequences (Pfam No: PF08460).

The effect of deleting *SMU.836* on the rate of cell lysis was determined as a mutation of *cbpD* in *S. pneumoniae* is known to cause reductions in cell lysis under some conditions (Guiral *et al.*, 2005; Kausmally *et al.*, 2005; Havarstein *et al.*, 2006). Deletion of *SMU.836*, however, had no measurable effect over and above the inherent variability in the slow rate of lysis of wild-type *S. mutans*, the lysis of which appeared to depend upon the culture $OD_{600}$ at the time of harvest (data not shown).

## DISCUSSION

The current study was undertaken to ascertain which of the proteins previously found to be upregulated in *S. mutans* biofilms (Rathsam *et al.*, 2005a,b) were associated with competence. Of the nine mutants that were constructed, deletion of *cysK*, *SMU.961* and *SMU.209*, which possessed no known

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Ssa.0036      NYAAANATTYPVGQCTWGAKALAP-----WAGNYWGNGGQWAASARRAGFRTGSTPEVGA
Sgo.2094      NHTTDPSNTYPVGECTWGAKALAP-----WAGNWWGNGGDWAASARRAGFRVGSTPQVGA
Smu.836       -------NTYPVGQCTWGVKELAP-----WIPNWLGNGGQWASTVAVKGFKIGTVPKVGA
CbpD          -QEIDQWRMYSR-QCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS
Consensus              Y   +CT                  GN   W       G++    P +G+

Ssa.0036      IACWDDGGYGHVGVVTHVESNTRIQIQESNYLGKQYISNFRGWFDPTASYWGRLTYI
Sgo.2094      IACWTDGGYGHVGVVTHVDSHKRIQIQESNYAGQRYIANFRGWFDPTIAQGT-----
Smu.836       IACWSDGGYGHVAYVTHVESNNRIQVKEANYKNQQYISNFRGWFDPTTSYLG-----
CbpD          ITWSTAGTYGHVAWVSNVMG-DQIEIEEYNY-GYTESYNKRVIKANTMTGFI-----
Consensus     I     G YGHV  V++V    +I+++E NY       N R     T
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**Figure 3** Sequence alignment of the putative CHAP domains of the proteins Ssa.0036 from *Streptococcus sanguinis* (residues 535–646), Sgo.2094 from *Streptococcus gordonii* (residues 436–542), Smu.836 from *Streptococcus mutans* (residues 437–536) and CbpD from *Streptococcus pneumoniae* (residues 63–170). The conserved cysteine and histidine residues are shown in bold.

competence promoter regions, did not alter transformation efficiency (Fig. 1, Table S4). This suggested that the three proteins encoded by these genes had no direct role in transformation, despite the result of a recent CSP-induced microarray analysis that suggested that *SMU.209* was induced by exposure to sCSP (Perry *et al.*, 2009).

Four of the nine mutants, the genes for recombinase A, *recA*, DNA processing protein, *dprA*, single-stranded DNA-binding protein, *ssbA*, and DpnII restriction endonuclease, *ssuRB*, encode proteins which are close homologues of proteins with known or proposed roles in competence in other bacteria (see Introduction and Table S1). The similarity of the transformation phenotypes of these *S. mutans* deletion mutants to those of *S. pneumoniae* and *B. subtilis*, support the supposition that these proteins possess the same function in *S. mutans* (Fig. 1, Table S4; Muckerman *et al.*, 1982; Alonso *et al.*, 1991; Martin *et al.*, 1995; Campbell *et al.*, 1998; Mortier-Barriere *et al.*, 1998; Berge *et al.*, 2002, 2003; Berka *et al.*, 2002; Ogura *et al.*, 2002; Lindner *et al.*, 2004; Tadesse & Graumann, 2007).

Deletion of the first of the remaining two genes that were studied, *SMU.769*, produced a slight but statistically significant decrease in chromosomal transformation (Fig. 1, Table S4), suggesting that Smu.769 may have a function in chromosomal integration of donor DNA. Smu.769 belongs to a family of proteins that are highly conserved in streptococci but for which no known function has been attributed (Table S1). Interestingly, the homologue to Smu.769 in *S. pneumoniae*, Sp.0782, does not affect chromosomal transformation (Peterson *et al.*, 2004). Whether this results from differences between the two species remains to be determined, though it has to be conceded that a direct role for Smu.769 in chromosomal transformation in *S. mutans* could not be verified in the current study because complementing the gene defect *in trans* was unsuccessful (Table S5).

In contrast to *SMU.769*, deletion of *SMU.836* affected both plasmid and chromosomal transformation, suggesting that Smu.836 is involved in an early stage of the transformation process before chromosomal integration (Fig. 1, Table S4). A direct role of Smu.836 in the transformation process was supported by the partial restoration of transformation by complementation with *SMU.836 in trans* (Fig. 2, Table S5).

*In silico* analysis of Smu.836 suggested that it belonged to a competence-induced family of peptidoglycan-degrading enzymes. *In silico* analysis also suggested that the choline-binding protein D, CbpD, of *S. pneumoniae* may be considered a potential member of this family despite having a different CWB domain to that in *S. mutans*, as it is the differences in the CWB domains that confer species specificity on these enzymes (Navarre & Schneewind, 1999; Anantharaman & Aravind, 2003). For example, the substrates of the pneumococcal-type CWB domain are the choline-containing ribotol teichoic and lipoteichoic acids that are uniquely found in the cell walls produced by *S. pneumoniae*, *Streptococcus oralis* and *Streptococcus mitis* species (Brundish & Baddiley, 1968; Gillespie *et al.*, 1993; Johnsborg *et al.*, 2008; Kilian *et al.*, 2008). Although the GBS Bsp-like repeats, found in *S. mutans*, are not similar to the pneumococcal CWB domain, they do contain conserved aromatic and charged residues suggesting a possible interaction with carbohydrate-containing polymers of the cell wall (Wren, 1991; Brown *et al.*, 2005).

In *S. pneumoniae*, mutation of *cbpD* has produced different results depending on the strain used. Studies in commonly used laboratory strains of *S. pneumoniae* have suggested that peptidoglycan hydrolases have no direct role in transformation (Peterson *et al.*, 2004; Kausmally *et al.*, 2005). However, this observation appears to result from multiple mutations in these *S. pneumoniae* strains arising from artificial selection for increased transformation with limited competence-associated autolysis (Avery *et al.*, 1944; Lanie *et al.*, 2007). Of particular relevance to the current observations is that changes in the *cbpD* gene have been noted in these laboratory strains (Lanie *et al.*, 2007). Contrary to results in the laboratory strains of *S. pneumoniae*, competence, specifically DNA binding and uptake in the progenitor of these laboratory strains, R36A, is affected by the absence of choline in its cell wall (Tomasz & Mosser, 1966; Tomasz, 1968; Tomasz *et al.*, 1971). This may suggest that the peptidoglycan hydrolases of *S. pneumoniae*, which contain CWB domain repeats (PF01473) specific for choline-containing ribotol teichoic and lipoteichoic acids, are essential for DNA uptake during competence. Supporting this conclusion is the observation that an insertion mutation of

*cbpD* in a clinical isolate of *S. pneumoniae*, G54, reduces plasmid transformation to <2% of that in wild-type (Rimini *et al.*, 2000), comparable to the result observed in the current study with the *SMU.836* mutant of *S. mutans*. Together, these results support the hypothesis that this family of peptidoglycan-degrading enzymes may play a role in the remodeling of the cell wall to permit the construction of DNA uptake machinery (Tomasz *et al.*, 1971; Dijkstra & Keck, 1996).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1** Homology of *Streptococcus mutans* LT11 proteins to other bacterial proteins.

**Table S2** Primers for polymerase chain reaction ligation mutagenesis and complementation.

**Table S3** Target gene primers used for polymerase chain reaction confirmation and creation of probes for Southern hybridization.

**Table S4** Efficiency of transformation for *Streptococcus mutans* LT11 and deletion mutants.

**Table S5** Efficiency of transformation for complemented *Streptococcus mutans* LT11 deletion mutants.

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