A tactile sensory system of *Myxococcus xanthus* involves an extracellular NAD(P)⁺-containing protein

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CsgA is a cell surface protein that plays an essential role in tactile responses during *Myxococcus xanthus* fruiting body formation by producing the morphogenic C-signal. The primary amino acid sequence of CsgA exhibits homology with members of the short-chain alcohol dehydrogenase (SCAD) family and several lines of evidence suggest that NAD(P)⁺ binding is essential for biological activity. First, the predicted CsgA secondary structure based on the 3 α /20 β -hydroxysteroid dehydrogenase crystal structure suggests that the amino-terminal portion of the protein contains an NAD(P)⁺ binding pocket. Second, strains with *csgA* alleles encoding amino acid substitutions T6A and R10A in the NAD(P)⁺ binding pocket failed to develop. Third, exogenous MalE-CsgA rescues *csgA* development, whereas MalE-CsgA with the amino acid substitution CsgA T6A does not. Finally, *csgA* spore yield increased ~20% when buffer containing 100 nM of MalE-CsgA was supplemented with 10 μ M of NAD⁺ or NADP⁺. Conversely, 10 μ M of NADH or NADPH delayed development for ~24 hr and depressed spore levels ~10%. Together, these results argue that NAD(P)⁺ binding is critical for C-signaling. S135 and K155 are conserved amino acids in the catalytic domain of SCAD members. Strains with *csgA* alleles encoding the amino acid substitutions S135T or K155R failed to develop. Furthermore, a MalE-CsgA protein containing CsgA S135T was not able to restore development to *csgA* cells. In conclusion, amino acids conserved in the coenzyme binding pocket and catalytic site are essential for C-signaling.

[Key Words: *Myxococcus xanthus*; short chain alcohol dehydrogenase; development; fruiting body formation; tactile response; cell-cell signaling]

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One of the most striking features of developing organisms is the spatial and temporal regularity with which complex multicellular structures emerge from a field of differentiating cells. Multicellular structures can result from spatially directed cell movement, spatially regulated cell division, or a combination of both. Myxobacteria are one of the simplest groups of organisms that form a multicellular developmental structure by directed cell movement (for review, see Shimkets 1990; Dworkin and Kaiser 1993). During myxobacterial development, ~10⁵ cells travel $\leq$ 200 μ m to assemble a fruiting body inside which they differentiate into dormant myxospores. Extracellular signaling molecules appear to coordinate the movement and differentiation of cells. Extracellular complementation of developmental mutants has provided evidence for five different extracellular signals (A through E) during *Myxococcus xanthus* development (Hagen et al. 1978; Downard et al. 1993). Mu-

tants that fail to produce one signal can be stimulated to develop by contact with wild-type cells or cells of a different complementation group. The A-signal consists of certain amino acids produced from proteolysis of extracellular protein (Kuspa et al. 1992a,b; Plamann et al. 1992). The E-signal may consist of one or more branched chain amino acids (Toal et al. 1995). The chemical nature of the remaining signals is unknown.

The C-signal is involved in a tactile signaling system initiated by contact between motile cells. Separation of *csgA*⁺ and *csgA*⁻ cells by a porous membrane blocks extracellular complementation suggesting that cell-cell contact is essential for C-signal transmission (Kim and Kaiser 1990b). Motility may be necessary to achieve optimal cell alignment for C-signaling because nonmotile cells are neither C-signal donors nor C-signal recipients (Kim and Kaiser 1990c). Mechanical side-by-side alignment of nonmotile cells restored C-dependent gene expression and sporulation suggesting improved C-signaling (Kim and Kaiser 1990d). C-signaling appears to be a venue through which cells synchronize their movement in response to cell-cell contact. Early in development cells organize themselves into approximately equidis-

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tant bands that move processively to give the appearance of traveling waves (Shimkets and Kaiser 1982). It has been proposed that oriented collisions between cells initiates C-signaling, which, in turn, causes changes in the motility reversal frequency (Sager and Kaiser 1994).

The *csgA* gene encodes a cell surface-associated protein responsible for C-signaling. Anti-CsgA antibodies inhibit both fruiting body morphogenesis and sporulation of wild-type cells (Shimkets and Rafiee 1990). Furthermore, anti-CsgA antibodies bind to the cell surface and extracellular matrix. Finally, CsgA purified from wild-type cells restores development when added to buffer covering a layer of *csgA* cells (Kim and Kaiser 1990a,b). Therefore, it was surprising to discover that CsgA contains consensus motifs found in the class II or short chain alcohol dehydrogenase (SCAD) family (Baker 1994; Lee and Shimkets 1994), as all the known members of the SCAD family are intracellular enzymes that catalyze reduction/oxidation reactions using either NAD⁺ or NADP⁺ (for review, see Reid and Fewson 1994).

We developed three models for the role of coenzyme in C-signaling. In the first model, NAD(P) binding is not required for C-signaling; CsgA is either a multifunctional protein or maintains the NAD(P) binding site as an unnecessary vestige of an ancestral protein. The most notable example of metabolic enzymes that have evolved structural roles without prior gene duplication are the vertebrate eye lens crystallin proteins (Wistow 1993). Some of the crystallins are metabolic enzymes that appear to have been recruited to their new function for reasons other than their catalytic activity. If this model is correct, then mutations that disrupt NAD(P)⁺ binding should not affect C-signaling. In the second model, coenzyme binding is not essential for C-signaling but protects the extracellular enzyme against UV light and oxidizing agents. The ability to bind NAD(P)H is thought to provide some protection against oxidation and UV irradiation in the vertebrate eye lens as a ζ crystallin protein lacking the coenzyme binding site is asso-

ciated with early onset cataract formation in humans (Borras et al. 1990; Huang et al. 1990; Rodriguez et al. 1992). According to this model, coenzyme binding should not be essential for C-signaling unless cells are exposed to UV light and oxidizing agents. The third model, in which coenzyme binding is essential for C-signaling, is unprecedented in that there are no known examples of extracellular cell-cell signaling proteins containing NAD(P)⁺ binding sites. Our results suggest that CsgA requires coenzyme for C-signaling and represents a novel type of cell-cell signaling system.

Results

CsgA contains a coenzyme binding motif

Site-directed mutagenesis was used to determine the *csgA* translation start site (Fig. 1). The start codon proposed by Hagen and Shimkets (1990) is GTG, which encodes formylmethionine when it serves as a start codon but otherwise encodes valine. Because valine can also be encoded by GTC, which is not normally a start codon, substitution of GTC for GTG should disrupt sporulation only if GTG serves as a start codon. If the start codon is further upstream, valine will be placed in the protein at the normal location and the change will be a silent mutation. Substitution of GTC for GTG did not disrupt sporulation indicating that the start codon is further upstream (Fig. 1). In the *csgA1070* allele a UGA stop codon was substituted for an arginine codon. This mutation abolished sporulation indicating that the start codon is still further upstream. In the *csgA1156* allele an ATG codon was replaced with a UGA stop codon, which disrupted sporulation, but similar substitutions 3 codons (*csgA1097*) or 30 codons (*csgA1096*) upstream did not disrupt sporulation. Therefore, the ATG codon disrupted in *csgA1156* is the most likely translational start codon. This start site is consistent with that predicted from pattern recognition analysis on the basis of third position GC bias (Bibb et al. 1984) and a codon usage table for *M. xanthus* (Shimkets 1993).

Allele	Sporulation
<i>csgA1096</i>	103%
<i>csgA1097</i>	92%
<i>csgA1156</i>	<1%
<i>csgA1070</i>	<1%
<i>csgA1073</i>	100%

Figure 1. CsgA translational start site determined by site-directed mutagenesis. The amino acid sequence of the entire open reading frame is given beginning with the first potential start codon. The numbers above the amino acid sequence refer to the amino acid position relative to the start codon deduced from this study. An asterisk refers to replacement of an existing codon with a stop codon. Each new *csgA* allele was integrated into the Mx8 *attB* site in strain LS523 (*csgA205*) by site-specific recombination. Cells containing each allele were examined for spore yield, which is given as percentage of DK1622 (*csgA*⁺)

sporulation. Cells containing alleles *csgA1070* (R52 → UGA stop codon), or *csgA1156* (M1 → UGA stop codon) failed to develop. However, substitution of the valine codon GTG with the valine codon GTC in the *csgA1073* allele did not affect development in the *csgA1096* [ATG → UAG stop codon] or the *csgA1097* [ATG → UGA stop codon] alleles. These results argue that the ATG codon referred to as M1 serves as the start codon.

The *csgA* translational start site is unusual in several regards. The *csgA* translational start site is not preceded by a consensus ribosome binding motif. Furthermore, the transcriptional start site (Li et al. 1992) is coincident with the translation start codon suggesting that the *csgA* mRNA lacks a ribosome binding site. It is relatively rare for an mRNA to lack a leader sequence, but ribosome binding sites are not essential for translation in *Escherichia coli* and other bacteria (Janssen 1993). When the mRNA lacks a leader sequence, a specific sequence near the 5' end of the coding region complementary to the 16S rRNA is used for translation initiation (for review, see Janssen 1993). In *csgA*, nucleotides 20–29 from the first nucleotide (A) in the translation initiation codon (5'-GAGCGAGCAG-3') show 70% complementarity to the *M. xanthus* 16S rRNA nucleotide sequence (Oyaizu and Woese 1985) between positions 1520 and 1529 (3'-CTAGGTTCGGC-5'). Nucleotides capable of hydrogen bonding are underlined. However, there is no direct evidence that the two nucleic acids hydrogen bond to stabilize *csgA* mRNA during translation. Another unusual feature of the translational start site is that the predicted CsgA amino terminus does not resemble a signal sequence typical of secreted proteins.

A MalE–CsgA fusion protein containing the entire CsgA-coding region was produced in *E. coli*, purified, and tested for biological activity by adding it to developing *csgA* cells submerged under a layer of buffer. The recombinant fusion protein produced fruiting bodies whose size and spatial distribution resembled wild-type fruiting bodies except for their nearly perfect hemispherical shape. Symmetrical fruiting bodies were described previously by Kim and Kaiser (1990a,b) when exogenous CsgA extracted from wild-type cells was added to *csgA* cells using the same assay. The fusion protein produced maximum spore levels at a concentration of ~100 nM although the spore yield was only ~80% that of wild-type cells (Fig. 2A). The presence of 10 μ M NAD⁺ in combination with 100 nM MalE–CsgA increased spore yield to wild-type levels (Fig. 2B). A similar level of stimulation was obtained with 10 μ M NADP⁺ (not shown). Conversely, 10 μ M NADH delayed development ~24 hr and slightly depressed spore levels (Fig. 2B). A similar level of inhibition was observed with 10 μ M NADPH (not shown). Neither 10 μ M NAD⁺ nor 10 μ M NADH affected fruiting body development or sporulation of wild-type cells (not shown).

CsgA mutations that abolish development

The crystal structures of two proteins related to CsgA are available, rat liver dihydropterine reductase (DHPR; Varughese et al. 1992) and 3 α /20 β -hydroxysteroid dehydrogenase (HSD; Ghosh et al. 1991, 1994). The structure of the more closely related HSD was determined previously at 2.6 Å resolution from crystals containing NADH (Ghosh et al. 1991). A refined structure is now available that shows remarkable three-dimensional similarity with DHPR (Ghosh et al. 1994). The secondary structural elements of CsgA and another *M. xanthus*

Figure 2. Rescue of *csgA* sporulation with exogenous MalE–CsgA fusion protein. (A) Rescue of *csgA* sporulation as a function of the MalE–CsgA concentration. Bars indicate the range of values from two experiments. (B) Rescue of *csgA* sporulation is influenced by the reduction/oxidation state of exogenous coenzyme. MalE–CsgA, 100 nM, was used to restore sporulation to unamended LS523 (*csgA205*) cells (□) or LS523 cells supplemented with either 10 μ M NAD⁺ (●) or 10 μ M NADH (○). Unamended DK1622 (*csgA*⁺) sporulation is shown for comparison (■). Values represent the average from two experiments.

SCAD member SocA (Lee and Shimkets 1994) were determined by aligning the deduced amino acid sequences of CsgA and SocA with HSD (Fig. 3). Amino acids conserved in the active site and coenzyme-binding pocket of other members of this family (Reid and Fewson 1994) are also observed in CsgA. In addition, all the structural elements found in HSD are present in CsgA with the exception the last β G sheet (Ghosh et al. 1994). Most of these structural domains contain conserved amino acids with the exception of β C and α D. A stereodiagram of the α carbon backbone of one subunit of the HSD was compared with that of CsgA generated with the molecular modeling program SIBYL. CsgA showed remarkable similarity to HSD in overall shape and the locations of the conserved amino acid residues lining the substrate and coenzyme binding pockets (not shown). An unusual fea-

Figure 3. Secondary structural information used for three dimensional model building. Alignment of CsgA and SocA with the 3 α /20 β -hydroxysteroid dehydrogenase (HSD) was performed using PILEUP (Devereux et al. 1984). Secondary structural motifs are shown below the sequence line based on the X-ray crystal structure of Ghosh et al. (1994). White letters in Black boxes indicate residues conserved in all three proteins. Numbers refer to amino acid positions in each protein.

ture of CsgA is observed in segment αF that contains amino acids forming a portion of the catalytic motif YXXXX (Ensor and Tai 1991, 1994; Albalat et al. 1992; Obeid and White 1992; Chen et al. 1993; White et al. 1994). CsgA has an AY repeat in this domain (Fig. 3). Although the YXXXX catalytic motif typical of other SCAD members is preserved if the sequence YRMSK is used in the alignment, the presence of these two additional amino acids in αF and the presence of R in the YXXXX motif is unique to CsgA.

The amino terminal βA - αB - βB - αC - βC - αD - βD - αE sequence is the coenzyme binding fold (Ghosh et al. 1991, 1994). The amino-terminal region of CsgA contains extensive primary and secondary homology with the HSD coenzyme-binding site prompting an examination of the role of this domain in CsgA signaling. In particular, HSD T12 may help stabilize the adenine-ri-

bose end of the dinucleotide in the coenzyme-binding pocket by hydrogen bonding (Ghosh et al. 1991, 1994). Site-directed mutagenesis was used to make an csgA allele encoding a T6A amino acid substitution that inactivated the gene (Fig. 4). The HSD residue R₁₆ side chain is likely to interact with the bis-phosphate moiety of the dinucleotide (Ghosh et al. 1994). A csgA mutation that encodes the R10A amino acid substitution also inactivated csgA (Fig. 4). The essential nature of these codons is further proof that the start codon is upstream as defined in Figure 1.

A similar strategy was used to examine the importance of the conserved catalytic site residues S135 and K155 (see Fig. 3). Modeling studies with HSD suggest that S139, Y152, and K156 side chains promote electrophilic attack on the C₂₀ carbonyl oxygen atom of cortisone, thereby enabling the carbon atom to accept a hydride from the reduced cofactor (Ghosh et al. 1994). Conservative substitution of the homologous amino acids from CsgA was used to examine their importance in development. The K found in the αF segment of the protein (see Fig. 3) is conserved in all SCAD members and is essential for catalytic activity (Persson et al. 1991; Reid and Fewson 1994). Chemical modification of this residue with diethyl pyrocarbonate inactivated 3 α ,20 β -hydroxysteroid dehydrogenase (Pasta et al. 1987). Furthermore, K156I substitution resulted in loss of catalytic activity of *Drosophila* alcohol dehydrogenase (Chen et al. 1993), as did the homologous K183R substitution in 11 β -hydroxysteroid dehydrogenase (Obeid and White 1992) and the K155L and K155Q substitutions in the human placental 15-hydroxyprostaglandin dehydrogenase (Ensor and Tai 1994). Site-directed mutagenesis was used to make a mutant csgA allele encoding the conservative amino acid substitution K155R that inactivated the gene (Fig. 4). Site-directed mutagenesis was also used to generate a mutant CsgA allele encoding the conservative amino acid substitution S135T with the resulting loss of activity (Fig. 4).

Several csgA alleles had been generated previously by mutagenesis of whole cells and we determined where the mutations were located. Allele csgA3330, created by uv light irradiation (Shimkets et al. 1983), may have been caused by an incorrectly repaired cytosine dimer, as adjacent G:C base pairs have been replaced with A:T base pairs. The base substitutions are in different codons resulting in amino acid substitutions M137I and G138S (Fig. 4). These amino acids are located in the region between βE and αF (see Fig. 3), which lines the catalytic pocket in HSD (Ghosh et al. 1991). csgA741 was isolated after mutagenesis with ICR-191 (Hagen et al. 1978) and contains a frameshift mutation in the region between βF and αG (Fig. 4), which lines the substrate binding pocket of HSD (Ghosh et al. 1991). csgA653 was isolated after mutagenesis with ethylmethane sulfonate (Hagen et al. 1978) and is a G:C $\rightarrow$ A:T transition that results in A157V (Fig. 4). This amino acid is located in the αF region of the protein (see Fig. 3) and is thought to be involved in subunit association. The homologous A159T mutation in *Drosophila* alcohol dehydrogenase elimi-

Figure 4. *csgA* amino acid substitutions encoded by various *csgA* alleles. Alleles *csgA1098* (T6A), *csgA1099* (R10A), *csgA1152* (D57N), *csgA1153* (S135T), *csgA1155* (K155R), *csgA1206* (R218N), and *csgA1051* (R218K) were generated by oligonucleotide-directed mutagenesis (Table 1). The remaining mutants were generated by mutagenesis of whole cells. Allele *csgA653* (A157V) was generated by ethyl methane sulfonate mutagenesis (Hagen et al. 1978). Allele *csgA3330* (M137I and G138S) was generated by uv light irradiation (Shimkets et al. 1983). Alleles *csgA731* and *csgA741* were generated with ICR-191 (Hagen et al. 1978) and encode an additional 176 and 220 amino acids, respectively. Alleles *csgA205* (T73 → stop codon) and *csgA278* (G217 → stop codon) were generated by Tn5 or Tn5 *lac* transposition, respectively, and encode sharply truncated proteins attributable to the presence of a stop codon at the transposon junction (Shimkets and Asher 1988). Alleles *csgA208* and *csgA269* were generated by Tn5 or Tn5 *lac* transposition, respectively (Shimkets and Asher 1988), and encode an additional 6 amino acids. Each substituted amino acid is shown below the wild-type amino acid sequence. Asterisks represent stop codons. All *csgA* alleles generated by oligonucleotide-directed mutagenesis were integrated into the Mx8 *attB* site in LS523 (*csgA205*) and assayed for sporulation. Cells containing each allele were examined for spore yield, which is given as percentage of DK1622 (*csgA*⁺).

	Allele	Sporulation
1 MRYVITGASRGIGFEFVQQLLRGDTVEAGVRSPEGARRRLEPLKQKAGNRLRIHALDVQD	<i>csgA1098</i>	<1%
A A	<i>csgA1099</i>	<1%
N	<i>csgA1152</i>	6%
61 DDSVRAFATNVCTGPFVDVLINAGVSGLWGCALGDVDYADMARTFTINALGPLRVTSAMLP	<i>csgA205</i>	<1%
*	<i>csgA208</i>	<1%
121 GLRQGALRRVAHVTSRMGSLAANTDGGAYAYRMSKAALNMAVRSMSDRLRPEGFVTLLH	<i>csgA1153</i>	<1%
T	<i>csgA1155</i>	<1%
IS	<i>csgA3330</i>	<1%
R	<i>csgA653</i>	<1%
V	<i>csgA269</i>	<1%
LTIIHK*		
181 PGWVOTDMGGPDATLPAVDVRGMLRVIDGLNPEHSGRFFDYQGTVEVPW	<i>csgA741</i>	<1%
RLG...220AA	<i>csgA278</i>	<1%
*	<i>csgA731</i>	<1%
N	<i>csgA1206</i>	3%
K	<i>csgA1051</i>	40%
DRS...176AA		

nated the ability to form stable homodimers or heterodimers (Chenevert et al. 1995).

The carboxy-terminal region is involved in substrate binding in HSD. The configuration of the carboxy-terminal region of CsgA differs substantially from HSD in that it is shorter and lacks the HSD substrate-binding pocket residues D227, T244, and P246 to Y251 (Ghosh et al. 1991). Several *csgA* alleles contain mutations in the carboxyl terminus. *csgA731* (Hagen et al. 1978), is an ICR-191-induced frameshift mutation that occurs just 5 amino acids from the carboxyl terminus (Fig. 4). Several *csgA* alleles were generated by Tn5 or Tn5 *lac* insertion (Shimkets and Asher 1988). These insertions can result in sharply truncated proteins in the case of *CsgA205* and *CsgA278* because of the existence of a stop codon at the transposon junction, or proteins with 6 amino acid carboxy-terminal tails attributable to insertion in a different codon position in the case of *CsgA208* and *CsgA269* (Fig. 4). The most interesting of these is *CsgA278* which allows cells to ripple but not progress any further in the developmental pathway (Shimkets and Asher 1988). This insertion resulted in deletion of the carboxy-terminal 13 amino acids. Apparently a positively charged amino acid is required at position 218. A mutation that encodes R218N resulted in a loss of sporulation, whereas the R218K substitution resulted in only a modest decrease in spore production. The significance of this finding is unclear as the carboxy-terminal portion of CsgA is substantially different from HSD. One possibility is that R218 is involved in association with a cell surface component through charged pair interactions.

Assay of mutant proteins

The preceding results demonstrated that mutations in the putative coenzyme-binding fold and catalytic site

produce *csgA* alleles with no developmental activity. A limitation of this approach is the difficulty in conclusively demonstrating that the mutant protein product is produced and secreted properly. For this reason, mutant *csgA* alleles were used to produce MalE-CsgA fusion proteins and assayed for C-signal activity using the submerged culture assay. A MalE-CsgA fusion containing the CsgA T6A substitution was unable to stimulate *csgA* mutant development (Fig. 5A). Furthermore this mutant protein has a reduced capacity to bind radiolabeled NAD⁺ in a filter binding assay (Fig. 5B), suggesting that T6 is essential for NAD⁺ binding. Together, these results argue that NAD⁺ binding is essential for biological activity.

A MalE-CsgA fusion protein containing the S135T amino acid substitution was unable to stimulate *csgA* development in submerged culture (Fig. 5A). This substitution is predicted to interfere with catalytic activity of SCAD members (Ghosh et al. 1991; 1994).

Survey of SCAD family members

SCAD family members catalyze reduction/oxidation reactions of a wide variety of ketones, aldehydes, and alcohols. Some SCAD substrates or products are signaling molecules in other organisms including steroids and prostaglandins in vertebrates, and nodulation factors in *Rhizobium* (Debelle and Sharma 1986). In addition, members of this family whose function is as yet unknown are involved in *Anabaena* heterocyst differentiation (Black and Wolk 1994), mouse adipocyte differentiation (Wenz et al. 1992), and sex determination in maize (DeLong et al. 1993). The SCAD family is also involved in the production of numerous secondary metabolites, including polyketide antibiotics (Bibb et al.

Figure 5. CsgA amino acid T6A substitution in the coenzyme binding domain produces biologically inactive protein and disrupts NAD^+ binding. Photographs of developing *csgA* cells supplemented with 100 nM MalE-CsgA. (A) *csgA* cells plus wild-type MalE-CsgA; (B), *csgA* cells plus MalE-CsgA T6A; (C), and *csgA* cells plus MalE-CsgA S135T. Bar, 200 μm . (D) NAD^+ filter binding assay. MalE-CsgA, 2 μM , was incubated with various concentrations of ^{32}P -labeled NAD^+ : wild-type MalE-CsgA (●) or MalE-CsgA T6A (■).

1989; Sherman et al. 1989), aflatoxins (Trail et al. 1994), and fungal melanin (Vidal-Cros et al. 1994). 11-*cis* retinol dehydrogenase is involved in the oxidation of retinol to retinaldehyde during mammalian visual chromophore synthesis (Chai et al. 1995; Simon et al. 1995).

The SCAD members most closely related to CsgA were grouped according to the type of substrate they use (Fig. 6). The amino acid identities and similarities with CsgA were calculated based on pairwise comparisons. Amino acid identity to bovine retinol dehydrogenase was the highest followed by a putative *Saccharomyces* protein of unknown function. Homology of CsgA with rat fatty acid synthase and human NADPH-dependent 15-hydroxyprostaglandin dehydrogenase was also striking in view of the large phylogenetic distance with *M. xanthus*.

Discussion

Contact between *M. xanthus* cells activates the C-signaling pathway (Kim and Kaiser 1990c,d) which in turn controls cell reversal frequency (Sager and Kaiser 1994) and regulates gene expression (Kroos and Kaiser 1987). Little is known about sensory transduction of tactile in-

formation among the prokaryotes. Precise localization of the translational start site suggests that *csgA* encodes a 24.5-kD protein with extensive amino acid identity to the SCAD family. Because CsgA is the only extracellular member of this family, it was of interest to determine whether coenzyme binding is essential for C-signaling. Previous studies demonstrated that a truncated form of CsgA lacking the coenzyme binding motif did not restore development to *csgA* mutants (Shimkets and Rafiee 1990). In the present study, addition of a MalE-CsgA fusion protein containing the entire CsgA protein-coding region restored development to *csgA* cells submerged under a layer of buffer. Presumably the protein self-assembles on the *csgA* cell surface to reconstitute wild-type-like cells. Cells with mutant *csgA* alleles encoding amino acid substitutions T6A and R10A in the NAD^+ -binding pocket failed to develop. Furthermore, a CsgA protein containing the amino acid substitution T6A failed to bind NAD^+ in vitro and did not restore *csgA* mutant development. Together these results argue that NAD(P)^+ binding is critical for C-signaling in vivo and in vitro.

There are several possible roles the dinucleotide could play in C-signaling. The most obvious is that of a hydride acceptor during chemical catalysis of an unknown substrate by CsgA. This notion is consistent with stimulation of CsgA-induced sporulation by 10 μM NAD^+ and NADP^+ and inhibition by NADH and NADPH. In the second type of model coenzyme modified by CsgA generates the signal. The possibilities are somewhat limited, as NAD^+ , NADP^+ , NADH, or NADPH did not rescue *csgA* development in the absence of exogenous CsgA. However, a more unusual CsgA-dependent modification of the coenzyme remains a possibility. Group A streptococci produce a cell surface-associated glyceraldehyde-3-phosphate dehydrogenase that also catalyzes ADP ribosylation in vitro (Pancholi and Fischetti 1993). The C-signal could be generated by covalent modification of a target protein by ADP ribosylation. However, unlike the *Streptococcus* protein, which belongs to a different class of dehydrogenases, CsgA did not catalyze auto ADP ribosylation in vitro (not shown). In the third type of model a conformational switch in CsgA may occur attributable to binding of coenzyme or coenzyme plus substrate that initiates the signaling cascade.

The discovery that CsgA has highest homology with bovine retinol dehydrogenase was surprising as retinol had not been reported previously in the myxobacteria. *Myxococcus fulvus* produces ≥ 60 different carotenoids (Reichenbach and Kleinig 1984), therefore, it is possible that the putative CsgA substrate is a polyisoprenoid. Steroids have been identified in the myxobacterium *Nanocystis exedens* but have not been reported in *M. xanthus* (Kohl et al. 1983). Members of two other SCAD substrate classes, carbohydrates (Fig. 6B) and acyl compounds (Fig. 6C), could be considered potential substrates because of their location on the cell surface. A number of polysaccharides are associated with the cell surface; however, the overall homology with enzymes using carbohydrate substrates is lower than any other

Enzyme	Accession #	Organism	S	I
A. Phenolic substrates				
2,3-Dihydro-2,3-dihydroxy-benzoate dehydrogenase	P15047	<i>Escherichia coli</i>	50.7	27.0
Biphenyl dihydrodiol dehydrogenase	P08694	<i>Pseudomonas pseudoalcaligenes</i>	49.0	25.9
cis-1,2-dihydrobenzene-1,2-diol dehydrogenase	P08088	<i>Pseudomonas putida</i>	50.0	27.9
Polyhydroxynaphthylene reductase	L22309	<i>Magnaporthe grisea</i>	48.0	26.9
DoxE	S27635	<i>Pseudomonas sp.</i>	48.1	<u>29.2</u>
B. Carbohydrate substrates				
Sorbitol-6-phosphate dehydrogenase	P05707	<i>Escherichia coli</i>	47.1	21.5
N-acylmannosamine 1-dehydrogenase	P22441	<i>Flavobacterium sp.</i>	49.1	26.0
C. Acyl-CoA derivatives (Fatty acids/Polyketides/Poly-β-hydroxybutyrate/Prostaglandins)				
3-oxoacyl-[acyl carrier protein] reductase	P25716	<i>Escherichia coli</i>	51.6	26.5
	P12785	<i>Rattus norvegicus</i>	<u>55.3</u>	28.8
Acetoacetyl-CoA reductase	P14697	<i>Alcaligenes eutrophus</i>	46.6	26.0
	P23238	<i>Zoogloea ramigera</i>	51.6	22.4
Keto-acyl reductase	P16543	<i>Streptomyces violaceoruber</i>	48.8	26.5
	P16544	<i>Streptomyces coelicolor</i>	46.6	27.4
	P35320	<i>Streptomyces lividans</i>	<u>53.1</u>	28.3
NADH-dependent 15-hydroxy-prostaglandin dehydrogenase	P15428	<i>Homo sapiens</i>	49.5	28.1
NADPH-dependent 15-hydroxy-prostaglandin dehydrogenase	P16152	<i>Homo sapiens</i>	<u>52.7</u>	28.6
Norsolorbate keto reductase	L27801	<i>Aspergillus parasiticus</i>	52.0	28.2
D. Isoprenoid derivatives (Steroids, Retinol)				
7-α-hydroxysteroid dehydrogenase	P25529	<i>Escherichia coli</i>	45.5	25.0
3-α,20-β-hydroxysteroid dehydrogenase	P19992	<i>Streptomyces hydrogenans</i>	49.8	27.0
Bile acid 7-dehydroxylase	P07914	<i>Eubacterium sp.</i>	49.1	24.3
Estradiol 17-β-dehydrogenase	P14061	<i>Homo sapiens</i>	50.2	26.9
11-cis retinol dehydrogenase	X82262	<i>Bos taurus</i>	<u>55.0</u>	<u>34.7</u>
	L27429	<i>Rattus norvegicus</i>	51.6	29.0
E. Unknown substrates				
Adipocyte Differentiation 27	P08074	<i>Mus musculus</i>	49.5	25.7
Nodulation protein NodG	P17611	<i>Azospirillum brasilense</i>	47.7	23.6
Nitrogen fixation FixR	P05406	<i>Bradyrhizobium japonicum</i>	50.5	<u>31.4</u>
ORF YKL071w	Z28071	<i>Saccharomyces cerevisiae</i>	<u>56.3</u>	<u>33.2</u>
Heterocyst differentiation HetN	L27429	<i>Anabaena sp.</i>	49.3	26.9
TASSELSEED2	L20621	<i>Maize</i>	51.4	<u>29.5</u>
Nitrogen Fixation fix-23	X64131	<i>Rhizobium meliloti</i>	50.4	20.9

Figure 6. Comparison of the amino acid homology of CsgA to other SCAD family members grouped according to the class of substrate they use. Alignment was carried out using the computer program GAP (Devereux et al. 1984) with gap weight = 3.00 and length weight = 0.10. S indicates the percentage of amino acid similarity and I indicates the percentage of amino acid identity from pairwise alignments over the full length of the two proteins. The five most closely related sequences in each category are underlined.

category. On the other hand, the overall homology with enzymes using acyl substrates is high. Ethanol and the phenolic derivatives (Fig. 6A) are not likely to be CsgA substrates because they are not associated with the *M. xanthus* cell surface.

Because CsgA apparently requires NAD(P)⁺ for its extracellular activity, a mechanism for secretion of coenzyme or cosecretion with CsgA is necessary. One possibility is that extracellular coenzyme is provided by developmental autolysis. During development, a portion of the cell population is sacrificed (Wireman and Dworkin 1977) and may release coenzyme.

The discovery that CsgA requires coenzyme binding for biological activity will likely lead to a radical shift in

thinking about the mechanism of C-signaling. Indeed, the mechanism is likely to be unique among known signaling systems; no other known signaling system involves a cell surface NAD(P)⁺ binding protein.

Materials and methods

Bacterial strains

M. xanthus cells were grown at 32°C in CTT broth or on CTT agar (Hodgkin and Kaiser 1977), supplemented, when necessary, with kanamycin sulfate (40 μg/ml). Strain DK1622 is CsgA⁺ and is used as the wild-type (Shimkets and Kaiser 1982). LS523 (CsgA205) is a csgA null mutant generated by transposon insertion (Shimkets and Asher 1988) (Fig. 4). *E. coli* was grown in LB

or LB agar supplemented with kanamycin sulfate (25 µg/ml), ampicillin (50 or 100 µg/ml), or tetracycline (12.5 µg/ml), when necessary.

For development, cells were harvested from CTT broth in the mid-exponential phase of growth (3×10^8 to 5×10^8 cells/ml), washed and resuspended in TPM buffer [10 mM Tris-HCl (pH 7.6), 1 mM K_2HPO_4 , 8 mM $MgSO_4$]. Cells, 5×10^8 , were spread on TPM agar (TPM buffer plus 1.5% Difco agar) and incubated at 32°C for 5 days. Then cells were removed from the surface of the agar plates and subjected to sonication at 50 µW for 30 sec (W380, Heat Systems Ultrasonics) to lyse vegetative cells and disperse spores. The myxospores were counted in a Petroff-Hausser counting chamber using phase-contrast microscopy.

Site-directed mutagenesis

Site-directed mutagenesis was performed by unique site elimination (USE) (Deng and Nickoloff 1992) on pLJS43, a pUC19-based plasmid containing the entire wild-type *csgA* gene. A list of mutagenic primers is given in Table 1. Mutations were confirmed by dideoxy sequencing (Sanger 1977) with *Taq* polymerase. Mutagenized *csgA* genes were subcloned into pJR25 and electroporated into LS523 with selection for kanamycin resistance. This plasmid does not replicate in *M. xanthus* cells. However, it contains the bacteriophage Mx8 attachment site *attP* and integrates into the chromosomal *attB* site by site-specific recombination (Li and Shimkets 1988).

DNA and amino acid sequence data were analyzed with the University of Wisconsin Genetics Computer Group sequence software package (Devereux et al. 1984). Secondary and tertiary structures of SocA and CsgA were modeled by using the computer program SIBYL (Tripods, Inc.) based on the crystallographic structure of the $3\alpha/20\beta$ -hydroxysteroid dehydrogenase (Ghosh et al. 1991; 1994).

Construction and purification of the MalE-CsgA fusion proteins

Plasmids containing MalE-CsgA translational fusions with wild-type or mutant *csgA* alleles were constructed in several steps. First, the polymerase chain reaction (PCR) was used to produce a *csgA* gene with appropriate restriction sites at either end. Primer 5'-GCAAAATATTATGCGCTACGTCATC-3' contained an *SspI* restriction site and primer 5'-GCAAAAGCT-

TGTGGAGCAGAAGTAC-3' contained a *HindIII* restriction site (both restriction sites are underlined). Plasmids containing the wild-type allele or a mutant *csgA* allele (T6A or S135T) were linearized by *HindIII*. The PCR mixture contained 100 ng of linearized plasmid, 200 nM of each primer, 200 µM each of dATG, dCTP, dGTP, and dTTP, 10 units of Stoffel fragment (Perkin-Elmer Cetus), 10 µM of KCl, 10 µM of Tris-HCl (pH 8.3), 4 µM $MgCl_2$ in a total volume of 100 µl. Mineral oil, 100 µl was placed on top of each reaction mixture. The PCR conditions were initial denaturation (98°C for 2 min), 4 preamplification cycles (96°C for 1 min, 42°C for 1 min, 72°C for 1.5 min), 20 amplification cycles (96°C for 1 min, 55°C for 1 min, 72°C for 1 min), and a final extension (72°C for 10 min). Each PCR product was digested with *SspI* and *HindIII* and then ligated to *XmnI*- and *HindIII*-digested pMAL-c (New England Biolabs). Each ligation mixture was transformed into *E. coli* XLI-Blue MRF' strain (Stratagene) and/or *E. coli* BL21 strain (Novagene) with selection for ampicillin resistance. The MalE-CsgA fusion junction was confirmed by dideoxy sequencing with *Taq* polymerase. Finally, the PCR-generated *csgA* structural gene (from *EcoRI* to *HindIII*) was replaced with the template *csgA* DNA fragment to eliminate the possibility that mutations occurred during PCR amplification.

Each MalE-CsgA fusion protein was purified from *E. coli* BL21 cells cultured in 160 ml of LB broth containing 0.2% (wt/vol) of glucose, 100 µg/ml of ampicillin, and 0.3 µM IPTG for 2 hr at 22°C. Cells were harvested by centrifugation and resuspended in 5 ml of buffer containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. Resuspended cells were disrupted by sonication on ice at 50 µW for eight 15-sec intervals (Heat Systems Ultrasonic) and subjected to affinity chromatography on an α -amylose column (New England Biolabs). About 600 µl of affinity-purified fusion protein was dialyzed (Spectrum; molecular mass cutoff = 12–14-kD) against 1000 ml of 10 mM MOPS (pH 7.2), 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 50 mM NaCl for 10 hr at 4°C and aliquots of dialyzed fractions were stored at 4°C for use within 24 hr or frozen at –70°C. The MalE-CsgA protein was stable ≥ 2 weeks at –70°C. Protein concentration was determined using the BCA reagents (Pierce) with bovine serum albumin standards. Each MalE-CsgA fusion protein was tested to determine whether it restored development to LS523 using the assay described by Kim and Kaiser (1990a,b). NAD⁺, NADP⁺, NADH, or NADPH (Sigma Chemical) was added at a final concentration of 10 µM.

Table 1. Primers used for site-directed mutagenesis

Mutagenic primers ^a	Codon or amino acid substitution	Allele number
GGATGCACCACTAGCCGAGGACAAC	ATG → UAG	<i>csgA1096</i>
GCATGCATGGTCATAACGCGCTG	ATG → UGA	<i>csgA1097</i>
ATGACGTAGCGTCAGCATGGCATTAAAC	M1 → UGA	<i>csgA1156</i>
CCCTGCTCGCGCCGGCGATGACGTAGC	T6A	<i>csgA1098</i>
CGAAACCAATGCCGGCGCTCGCTCCG	R10A	<i>csgA1099</i>
GCGCATGGATTACAGGCGGTTGC	R52 → UGA	<i>csgA1070</i>
CTGTCTGTCGTCACGTTTCAGCGCATGG	D57N	<i>csgA1152</i>
CGAACGCGCGGACGCTGTCGTCG	V64V (GTG → GTC)	<i>csgA1073</i>
AACGAGCCCATACGCGTGGTGACGTGC	S135T	<i>csgA1153</i>
TTCAGCGCGGCTCGCGACATGCGG	K155R	<i>csgA1155</i>
GCACAGCGGCAACTTCTTCGAC ^b	R218N	<i>csgA1206</i>
GTCGAAGAACTTGCCGCTGTGC	R218K	<i>csgA1051</i>

^aPrimers are coding strand sequences (i.e., complementary to mRNA sequences) and are given 5' → 3'. Boldface letters refer to the *csgA* base substitutions.

^bPrimer is noncoding strand sequence and is given by 5' → 3'.

Fruiting bodies were photographed with a Wild MPS51 dissecting microscope.

Cofactor binding assay

Binding of NAD⁺ to MalE-CsgA was performed using a filter binding assay (Mukherjee et al. 1993). Ten microliters of binding buffer [10 mM MOPS (pH 7.2), 1 mM CaCl₂, 4 mM MgCl₂, and 500 mM NaCl] containing 2 μ M fusion protein and various concentrations of radiolabeled NAD⁺ was incubated for 1 hr at 25°C. NAD⁺ labeled with ³²P (~1000 Ci/mmol; Amersham) was diluted with NAD⁺ (Sigma) to a specific activity of ~1.0–2.0 Ci/mmol. Then protein plus NAD⁺ was applied to a nitrocellulose filter (Millipore; pore size, 0.022 μ m) under vacuum. The filter was washed twice with 3 ml of binding buffer under vacuum and air dried. Radioactivity was measured with a LS3801 liquid scintillation counter (Beckman). Bovine serum albumin was used in measuring background levels of NAD⁺ association, and the background levels were subtracted from the experimental values.

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