Bypass of A- and B-Signaling Requirements for *Myxococcus xanthus* Development by Mutations in *spdR*

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Mutations in *spdR*, previously reported to bypass the developmental requirement for B-signaling in *Myxococcus xanthus*, also bypass the requirement for A-signaling but not C-, D-, or E-signaling. Mutations in *spdR* restored nearly wild-type levels of sporulation to representative A-signal-deficient mutants carrying asgA476, asgB480, and asgC767 and improved the quality of fruiting body formation in the asgB480 mutant. The defect in A-factor production by the asgB480 mutant was not restored in the *spdR*2134 asgB480 double mutant.

*Myxococcus xanthus* is a gram-negative bacterium that undergoes a primitive multicellular developmental process (5). In response to starvation, cells aggregate to form macroscopic fruiting body structures containing a few hundred thousand cells. Within the fruiting body, a portion of the cells differentiate into environmentally resistant myxospores. When nutrients become available, the spores germinate to form vegetatively growing cells, and the cycle repeats.

The developmental process is characterized by a temporal cascade of development-specific gene expression, which is absolutely dependent upon cell-cell signaling (9). Five classes (A to E) of intercellular signaling mutations have been identified, designated asg for A-signal, bsg for B-signal, etc., each of which arrests development at a characteristic stage (4, 7, 9, 12). Each class of signaling mutant is thought to be defective in the generation of a distinct extracellular signal that is required for continued progress through the developmental program.

In a genetic approach to identifying additional components of the bsg-dependent regulatory pathway, bypass suppressors were previously identified which allow *bsgA* mutants to form mature fruiting bodies containing nearly normal numbers of viable, sonication-resistant myxospores (5; J. Cusick and R. E. Gill, submitted for publication). Among the suppressors that bypassed the *bsgA* requirement were those that inactivated *spdR*, a gene that encodes an NtrC-like component of a two-component regulatory system (8).

**Mutations in *spdR* suppress the developmental defect of an A-signaling mutant.** To further characterize the relationship between *spdR* and intercellular signaling during development, the *spdR*2134 mutation was introduced into a representative of each of the five classes of signaling mutant [DK480 (asgB480), M853 (bsgA330), LSS23 (csgA205), DK429 (dsg429), and JD258 (csgA4)] (2, 4, 13). The *spdR*2134 mutation was introduced into these strains using homologous recombination to integrate plasmid pREG2134, which contains the internal *NeoI* fragment from within the *spdR* coding sequence (8) cloned into the EcoRI site of pCR2.1 (Invitrogen). Plasmid integration was predicted to disrupt the *spdR* gene, resulting in strains that contained only two truncated, nonfunctional copies of *spdR*. This genotype was confirmed by Southern blot analysis (data not shown). The transcriptional organization of the *spdR* locus has not been determined, and so the possibility that the *spdR*2134 mutation may have a polar effect on as yet unidentified downstream genes cannot be ruled out.

If the *spdR* mutation in these strains bypassed the signal requirement, then one would expect an increased efficiency of development in the double mutant compared to its nondeveloping parent. Development was induced by concentrating exponentially growing cells to a density of $5 \times 10^9$ cells per ml in TPM (10 mM Tris-hydrochloride [pH 7.6], 1 mM KH$_2$PO$_4$, 8 mM MgSO$_4$), and spotting 10-μl spots onto TPMP agarose medium (TPM supplemented with 0.1% sodium pyruvate and solidified with 1% [wt/vol] electrophoresis-grade agarose [Bio-Rad]). Media solidified with agarose were used for assessing the phenotype of asg mutants and their derivatives because certain asg mutants have a particularly high background level of sporulation on medium containing the standard bacteriologic grade of agar.

Duplicate plates containing 10 spots each were prepared for each strain and incubated at 30°C for 7 days. Spots were gently scraped from the agar surface, resuspended in 1 ml of TPM buffer, and sonicated. Refractile spores were counted microscopically using a Petroff-Hauser counting chamber. The numbers of viable spores were determined by plating on nutrient CTT medium after heating to 50°C for 2 h to kill vegetative cells and sonicating to disperse the surviving spores. Numbers of refractile and viable spores were within 20% agreement.

For the *bsg* mutant, the *spdR* *bsgA* double mutant produced mature fruiting bodies that were morphologically similar to those of wild-type cells and nearly wild-type numbers of viable spores. This result confirmed that the method used in these studies to disrupt *spdR* suppresses the B-signaling defect imposed by *bsgA*.

There was no observable effect on development when the *spdR* mutation was introduced into the C-, D-, or E-signaling mutants. However, the *spdR* mutation resulted in a dramatic improvement in the development of the asgA476 mutant, defective in A-signaling (Fig. 1). Morphologically, the fruiting bodies formed by the *spdR* asgA476 double mutant were con-
Considerably smaller and more numerous than those formed by the wild type or the spdR mutant itself, and a prominent ridge of fruiting bodging structures formed a ring at the outer margin of the spotted cell suspension. Fruiting bodies contained re-fractile spores; however, unusually large numbers of spores also formed in the cell mat outside of the fruiting bodies. The spdR mutation increased the numbers of spores produced by the asgB480 mutant by nearly 300-fold (average of two isolates) compared with the asgB480 mutant itself, and to greater than 50% of the number produced by wild-type or spdR mutant cells.

The spdR mutation did not correct the pigmentation or motility defect associated with asgB (data not shown). The spdR asgB double mutant retained the tan pigmentation and reduced swarming motility characteristic of the asgB parent. It is likely that the reduced motility contributes to the aberrant morphology and distribution of fruiting bodies produced by the double mutant.

There is mounting evidence that A-signaling is a far more complex regulatory step than originally envisioned (3, 6). To determine whether suppression of the asgB developmental defect by spdR is a common characteristic of other classes of A-signaling mutants, we determined the effect of spdR mutations on the behavior of mutants carrying two additional A-signaling mutations, asgA476 and asgC767 (Table 1). Introduction of the spdR2134 mutation into either the asgA476 or asgC767 mutant background restored the level of sporulation by more than 100-fold, to levels approaching that of the wild type (12.9 and 70.2% of wild-type level, respectively).

**Suppression of the developmental defect of asgB by mutations in spdR does not restore A-factor production.** Previous studies have shown that fruiting body formation, spore formation, and transcription of developmental genes by the asgB mutants are all restored by exogenous addition of conditioned starvation medium from wild-type cells undergoing development in suspension (11). The conditioned medium contains at least two active components, a heat-labile, trypsin-like protease activity, and a pool of peptides and amino acids (presumably the product of the protease activity), and are collectively known as A-factor. This activity can be mimicked by the addition of either a heterologous protease (e.g., trypsin) or an appropriate concentration of specific amino acids. A-factor activity is absent or drastically reduced in asgB mutants, including the asgB480 mutant used in these studies. However, the role of the asgB product in the synthesis or release of A-factor components has not been described.

It is possible that mutations in spdR bypass the requirement for asgB either by allowing release of authentic A-factor or by fortuitously allowing the release of an unrelated protease or pool of amino acids from the cell. In either case, a functional source of A-factor would be provided. To test this possibility, developmental supernatants of two spdR asgB double mutant isolates were assayed for A-factor activity, and the levels of activity were compared to those of the wild type and the asgB parent (Table 2). A-factor production was assayed as previously described (10, 11). Exponentially growing cells in CTT medium were washed and resuspended in MC7 at 5 × 10^9 cells per ml. The suspension was shaken vigorously at 32°C for 2 h. Cells were removed by centrifugation, and the supernatant was assayed for A-factor activity. A-factor activity was assayed by measuring the restored expression of the asg-dependent lacZ fusion Φ4521 by strain DK4324 (asgB480 Tn5 lacZΩ4521).

Transcription of this lacZ fusion occurs within the first hours of development in an A-signal-dependent fashion, and so occurs in asg^- cells or in asg mutants upon addition of an exogenous source of A-factor. Exponentially growing DK4324 test cells were washed and resuspended in MC7 at 5 × 10^9 cells per ml, and 25 µl of cell suspension was mixed with dilutions of conditioned medium and adjusted to a final volume of 400 µl. Suspensions were incubated for 20 h at 32°C, followed by determination of β-galactosidase activity (as a measure of expression of the Φ4521 lacZ fusion). One unit of A-factor activity was defined as the amount required to produce 1 U of

**TABLE 1. Restoration of spore formation of representative asg mutants by the spdR2134 mutation**

<table>
<thead>
<tr>
<th>Mutation(s)</th>
<th>Spores produced (% of wild-type level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (wild type)</td>
<td>100</td>
</tr>
<tr>
<td>spdR2134</td>
<td>93.2</td>
</tr>
<tr>
<td>asgA476</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>spdR2134 asgA476</td>
<td>12.9</td>
</tr>
<tr>
<td>asgB480</td>
<td>0.18</td>
</tr>
<tr>
<td>spdR2134 asgB480</td>
<td>54.9</td>
</tr>
<tr>
<td>asgC767</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>spdR2134 asgC767</td>
<td>70.2</td>
</tr>
</tbody>
</table>

* All strains are in the genetic background of the development-proficient strain DK101. Strains containing asg mutations were derived from DK476 (asgA476), DK480 (asgB480), and DK767 (asgC767), obtained from D. Kaiser (7). spdR mutations were introduced into DK101 or asg mutant strains by integration of plasmid pREG2134, containing an internal NcoI fragment of spdR. Cells were spotted onto TPM-agarose and incubated for 7 days at 30°C. The yield of spores is expressed as a percentage of the spores produced by the wild-type parent strain. The average yield of spores from the wild-type parent strain was 4.6 × 10^7 spores per plate (5 × 10^5 cells).
β-galactosidase (1 nmol of o-nitrophenyl [ONP] per min per 1.25 × 10⁶ cells).

A-factor activity was readily detectable in the wild-type developmental supernatant (12 U/ml of supernatant), but was present in supernatants of the parent asgB strain at only very reduced levels (0.8 U/ml, 7% of the wild-type activity). These values are comparable to those reported previously (11). When assayed under these conditions, the level of A-factor activity in developmental supernatants of the two spdR asgB double mutants (0.95 and 0.6 U/ml, respectively) was not significantly different from that of the asgB parent.

These data indicate that bypass of the developmental defect in the asgB mutant by the spdR mutation does not occur by release of either authentic A-factor or a surrogate activity that functionally substitutes for A-factor. Rather, the data suggest that mutations in spdR alter the regulatory pathways in early development, rendering them independent of the A-signaling event. Furthermore, when combined with the results of earlier studies (8), these results show that mutations that disrupt spdR bypass the developmental requirement for two genetically and biochemically distinct signaling steps in M. xanthus development and suggest the possibility that the A- and B-signaling pathways share regulatory components.

Interestingly, a second bsgA suppressor has recently been identified that bypasses the requirement for B- and C-signaling but not A-signaling (J. Cusick, E. Hager, and R. E. Gill, submitted for publication). These observations suggest two parallel pathways of regulation early in development, one requiring B- and C-signaling, and the other requiring B- and A-signaling.

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REFERENCES