A genetic link between light response and multicellular development in the bacterium *Myxococcus xanthus*

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The Gram-negative bacterium *Myxococcus xanthus* responds to blue light by producing carotenoid pigments (Car⁺ phenotype). Genes for carotenoid synthesis lie at two unlinked chromosomal sites, the *carC* and the *carBA* operon, but are integrated in a single “light regulon” by the action of common *trans*-acting regulatory elements. Three known regulatory genes are grouped together at the (light-inducible) *carQRS* operon. By screening the Car phenotype of a large collection of transposon-induced mutants, we have identified a new *car* locus that has been named *carD* (*carD1* for the mutant allele). The *carD* gene product plays a critical role in the light regulon, as it is required for activation of the *carQRS* and *carC* promoters by blue light. The *carD1* mutant is impaired in the (starvation-induced) developmental process that allows *M. xanthus* cells both to form multicellular fruiting bodies and to sporulate. Our results indicate that the *carD* gene product is also required for the expression of a particular set of development-specific genes that are normally activated through the action of intercellular signals.

[Key Words: Blue light; gene regulation; carotenoid genes; Myxobacteria; multicellular development; cell interactions.]

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Blue light modulates various physiological processes both in prokaryotic and eukaryotic microorganisms (Senger 1987). Little is known about the molecular mechanisms for reception and transduction of the light signal, but in a few cases the action of light has been associated with differential gene expression. This is well illustrated by the responses to light of the bacterium *Myxococcus xanthus* (see below), and the fungus *Neurospora crassa*. In the latter, both asexual differentiation and the synthesis of carotenes are regulated by blue light, which has been shown to transcriptionally activate several genes related to both processes (Nelson et al. 1989; Sommer et al. 1989; Schmidhauser et al. 1990; Lauter and Russo 1991; Lauter et al. 1992).

M. xanthus belongs to a group of Gram-negative, non-photosynthetic bacteria that can communicate intercellularly (Kaiser and Losick 1993). This ability allows them to respond to starvation conditions by building complex multicellular structures (fruiting bodies) where the vegetative cells differentiate into forms of resistance or myxospores. (For a recent compilation on the myxobacteria, see Dworkin and Kaiser 1993.) *M. xanthus* responds to illumination with blue light by producing carotenoids, which are known to play a protective role against photochemically generated oxidants (Rau 1988;

Bensasson et al. 1993). The study of this response by genetic and molecular means has revealed a complex network of regulatory actions that are set in motion by blue light and activate the carotenoid genes. This network (the light regulon) is outlined in Figure 1 (for review, see Hodgson and Murillo 1993).

All but one of the genes for carotenoid synthesis in *M. xanthus* are grouped together in a single, light-activated operon (the *carBA* operon) in which the genes necessary for the synthesis of phytoene (the first C₄₀ carotene precursor) are included, as well as the genes necessary for other steps downstream in the pathway. The unlinked gene *carC* encodes the enzyme that acts in the conversion of phytoene into neurosporene and is also activated by light at the transcriptional level. Contrary to that which happens with the *carBA* operon, the light induction of *carC* takes place only when the cells enter the stationary phase or are starved of carbon (Fontes et al. 1993; Ruiz-Vázquez et al. 1993).

The effect of light on the expression of the carotenoid genes is mediated by the action of four regulatory genes. Three of them, *carQ*, *carR*, and *carS*, are grouped together, in that order, in a single operon that is also activated transcriptionally by light. This operon is unlinked to the structural genes. Gene *carR* encodes a membrane-spanning protein and thus, might participate in the reception of the light signal. Lack-of-function mutations in *carR* cause the light-independent expression of all

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Figure 1. The light regulon of *M. xanthus*. Four unlinked loci (*carQRS*, *carD*, *carBA*, and *carC*) are represented. Discontinuous arrows connect some genes with their gene products. Continuous arrows indicate negative [(−)] or positive [(+)] effects on gene products or promoters (P). Blue light deactivates the membrane-bound protein CarR; therefore protein CarQ can stimulate the promoters of *carQRS* and *carC* through interaction with CarD. Activation of the *carQRS* operon results in high expression of CarS, which activates the promoter of the *carBA* operon. This operon contains several structural genes and the regulatory gene *carA*. This somehow mediates activation of the *carB* and the *carC* promoters. The product of *carB* participates in the synthesis of the first C₄₀ precursor, phytoene. This is converted into neurosporene (the first colored carotene) by the product of *carC*. Further processing of neurosporene depends on other *car* genes that are located between *carB* and *carA* and that form a single operon with these two genes. References for this model are indicated in the introductory section. The identity of *carD* and the interaction of CarD with CarQ to activate the *carQRS* and *carC* promoters are inferred from results included in this paper.

known *car* loci (the *carQRS* and *carBA* operons, and the *carC* gene), thus leading to the constitutive production of carotenoids. Lack-of-function mutations in *carQ*, which are epistatic over the *carR* mutations, have the opposite effect. They result in none of the *car* loci being activated by light and, therefore, in lack of carotenoid production. Lack-of-function mutations at gene *carS* block specifically light induction of the *carBA* operon (Martínez-Laborda et al. 1986; Martínez-Laborda and Murillo 1989; Fontes et al. 1993; Hodgson 1993; McGowan et al. 1993). The fourth regulatory gene, *carA*, forms a part of the *carBA* operon. It is defined by mutations that map at the 3' region of the gene cluster and have a dual effect on the light regulon. These mutations result in the light-independent expression of the *carBA* operon at a high level and are epistatic over the *carQ* mutations. The *carA* mutations also result in the re-

duced activation of *carC* by light (Martínez-Laborda and Murillo 1989; Fontes et al. 1993).

All indicated data lead to the model of Figure 1. In the dark, protein CarR blocks the action of CarQ; therefore, the *carQRS* operon is expressed at a low level. Light inactivates protein CarR, and CarQ is free (or active) to stimulate the expression of the *carQRS* operon. This results in a sufficient level of protein CarS being accumulated to activate the *carBA* operon. Data to be discussed here indicate that CarQ also activates gene *carC*. The action of CarQ and CarS on the *car* promoters is somehow mediated by the action of *carA*.

Sequence analysis of the *carQRS* gene products does not reveal any indication on operational mechanisms, apart from the membrane-spanning domain mentioned above for CarR. In particular, no amino acid stretches homologous to any of the various domains known to participate in the binding of proteins to DNA were detected in either CarQ or CarS (McGowan et al. 1993); thus, the possibility existed that other elements operating in the regulatory network had gone undetected. Therefore, we set about searching for new *car* genes. Here, we report the identification of a new locus, unlinked to all other *car* loci, that we have named *carD*. This locus plays a major role in the light regulon, as its mutations block the light induction of all *car* genes. Gene *carD* participates in other regulatory circuits controlling gene expression in *M. xanthus*. The *carD* mutant cells are blocked at an early stage of fruiting body formation, a phenotype that is associated with the lack of expression in those cells of a particular set of development-specific genes.

Results

A novel mutation in the light response of *M. xanthus*

Screening was carried out for Car mutations in populations of cells that had been mutagenized randomly with transposon Tn5 (resistance to kanamycin, Km^R). In a set of experiments, the standard strain DK1050 was mutagenized. DK1050 forms yellow colonies in the dark (attributable to an uncharacterized colored compound) that turn red after illumination for ~12 hr or more (attributable to the accumulation of carotenoids). Dark-incubated plates were used to screen ~3000 independent Km^R colonies for a constitutive mutant phenotype (Car^C, red colonies). The same plates were then illuminated for 24 hr and screened for a negative mutant phenotype (Car[−], yellow colonies). Two Car[−] mutants were detected in these experiments (named MR509 and MR510).

In a second set of experiments, the Car^C mutant strain MR151 (*carR3*) was mutagenized. MR151 forms red colonies both in the dark and in the light. About 8000 independent Km^R colonies were grown in the dark and screened for a yellow phenotype. Eight colonies were detected (named MR500, MR501, and MR503–MR508). All of them remained yellow when grown in the light; therefore, each of the mutants must carry an epistatic Car[−] mutation rather than a suppressor mutation of *carR3*.

Previously characterized Car^- mutations lie at three unlinked genes: *carQ*, which codes for an activator of the carotenoid genes, and *carB* and *carC*, each of which encodes a different enzyme for the synthesis of neurosporene, the first colored carotene precursor (Fig. 1). Km^R was transduced separately from each of the new Car^- mutants to *M. xanthus* strains carrying a tetracycline-resistant (Tc^R) marker at (or closely linked to) one of the *car* genes indicated. According to the segregation data for Km^R and Tc^R (not shown), seven of the new Tn5 insertions were located at *carB*, and another at *carC*. However, the Km^R markers in strains MR508 and MR509 were unlinked to any of the tested *car* loci. When Km^R was transduced from MR508 and MR509 back to the parental strain, all checked transductants (>500) were Car^- . Therefore, the mutant phenotype was attributable in both cases to the transposon insertion and was not the consequence of secondary mutations.

After in situ replacement of Tn5 by Tn5-132 in MR509, we found the two new *car* sites to be unlinked to each other (data not shown). The Tc^R derivative of MR509 was named MR522. Further data to be presented here correspond to the *car* locus altered in MR509. This locus and its mutant allele were named *carD* and *carD1*, respectively, whereas the Tn5 (Tn5-132) insertion site was named ΩMR509 . We checked for the accumulation of carotenoids and their carotene precursors in MR509, but none was detected either in dark- or in light-grown cultures.

Mutation *carD1* prevents light activation of the *car* genes

Mutation *carD1*, originally isolated in the Car^+ strain DK1050, acted epistatically over a Car^C mutation at *carR*. When MR151 (*carR3*) was used as recipient for

transduction of $\Omega\text{MR509}::\text{Tn5}$, all of the Km^R transductants showed a Car^- phenotype.

The *carA* mutant strain MR7 (*carA1*) forms orange colonies in the dark, as *carA1* causes constitutive expression of the carotenoid genes present in the *carBA* operon. This effect, added to the basal expression of *carC*, causes the formation of some colored carotenoids. The end product of the pathway, however, is not accumulated at the high level found in a *carR* mutant. MR7 colonies turned red in the light because of activation, although at a reduced level, of the *carC* gene (Martinez-Laborda et al. 1990; see introductory section and Fig. 1). When $\Omega\text{MR509}::\text{Tn5}$ was transduced into MR7, all Km^R transductants showed the same orange phenotype as the parental strain in the dark, although none of them turned red with illumination. Thus, *carD1* is not epistatic over *carA1* for the effect on the *carBA* operon but seems to block light activation of *carC*.

M. xanthus strains, which carry gene *lacZ* fused transcriptionally to the promoter of *carQRS*, *carB*, or *carC*, have been obtained (Balsalobre et al. 1987; Fontes et al. 1993; Hodgson 1993). To understand better the function of *carD*, mutation *carD1* was transferred to each of these strains. The parental strains and their *carD1* derivatives were then used for the analysis of β -galactosidase expression in dark- and light-grown cultures. As seen in Figure 2, *carD1* completely inhibited light activation of all tested promoters. These experiments were repeated with strains that strongly expressed the same set of *lacZ* fusions in the dark, because of the simultaneous presence of a *carR* mutation. Mutation *carD1* also inhibited the light-independent expression of the tested promoters that is normally found in a *carR* mutant background (Fig. 2). In all of these experiments, *carD1* was transduced into the recipient strains by selection of the associate antibiotic resistance marker. Note that all data in Figure

Figure 2. Effect of a *carD* mutation on the expression of different *car* genes. At time zero cell samples growing exponentially in the dark were divided in two, one for the dark (closed symbols) and the other for the light (open symbols). Samples in III and VI were at the late exponential phase and reached the stationary phase at the time indicated by the arrows. Data are β -galactosidase-specific activities from *lacZ* transcriptional fusions (arrows at top) to *carQ* (I,IV), *carB* (II,V), and *carC* (III,VI). Strains are (I) MR397 (■,□) and its *carD*::Tn5-132 derivative MR565 (●,○); (II) MR418 (■,□) and its *carD*::Tn5 derivative MR518 (●,○); and (III) MR553 (■,□) and its *carD*::Tn5-132 derivative MR583 (●,○); (IV) MR398 (■) and its *carD*::Tn5-132 derivative MR566 (●); (V) MR406 (■) and its *carD*::Tn5-132 derivative MR559 (●); and (VI) MR555 (■) and its *carD*::Tn5-132 derivative MR582 (●). IV–VI are with dark-grown samples only.

2 were obtained with a randomly picked colony from >50 sib transductants. All exhibited the same behavior when tested on light- or dark-grown X-gal plates, failing to develop the blue color indicative of a significant production of β -galactosidase.

Because the activation of *carB* by light depends on gene *carS* being expressed at a high level, the negative effect of mutation *carD1* on the expression of *carB* could be attributable to the reduced expression of *carQRS*. Mutant conditions are known in which transcription of *carB* does not depend on light activation of the *carQRS* operon. One such condition involves the *carA1* mutation. Another condition corresponds to the transposon insertion Ω DK1910::Tn5 (Tn5-132), which is close to, but does not interrupt, the *carQRS* operon. An outward promoter activity in the IS50L element at the end of the transposon results in *carQRS* being transcribed at high level in the absence of light. As a consequence, *carB* is also expressed at high level in the dark (Balsalobre et al. 1987; Hodgson 1993). In accordance with the epistatic relationship described above, *carD1* did not prevent the high expression of *carB* in the dark caused by mutation *carA1*; nor did it prevent the IS50L-dependent expression of *carQRS*, or the ensuing high expression level of *carB* in the dark (Fig. 3). We should note that this latter experiment was carried out with a different *carD* mutant allele, named *carD2*, that behaved exactly as *carD1*. Allele *carD2* is a frameshift mutation generated after cloning the *carD* locus (to be described below) and is not associated with any antibiotic resistance marker. Therefore, we could use the *carD2* mutant strain as recipient for the transduction of the Tn5-*lac* expression probe at *carB* (selecting for Km^R) or the insertion Ω DK1910::Tn5-132 (selecting for Tc^R), or both. Altogether, our data indicate that the negative effect of a *carD* mutation on the expression of *carB*, in an otherwise wild-type genetic background, is a consequence of the blocking effect of that mutation on the expression of *carQRS*.

We checked whether lack of *carC* expression in the *carD* mutants was also attributable to decreased *carQRS* expression by testing the effect of the Tn5 insertion that expresses *carQRS* constitutively. As shown in Figure 3, mutation *carD2* prevented light activation of *carC* even in the presence of the indicated Tn5 insertion. Thus, the negative effect of a *carD* mutation on *carC* is not simply a consequence of that mutation reducing the expression of *carQRS*.

Mutation carD1 prevents fruiting body formation and blocks expression of several developmentally activated genes

As deduced from the microscopic observation of colony edges (not shown), strain MR509 is not strongly impaired in gliding, the motility system that is typical of the myxobacteria and other groups of bacteria (for review, see McBride et al. 1993). MR509 was, however, unable to form the multicellular fruiting bodies built by wild-type cells on starvation plates. Most studies on multicellular development in *M. xanthus* have used DK1622 as the

Figure 3. Differential regulation of the *carB* and *carC* promoters. Data and symbols are as in Fig. 2. Drawings at the top depict the *car* gene that is fused to the *lacZ* probe (big arrow) and other relevant features of the corresponding strain. (I) Strain MR609 (■, □) and its *carD2* derivative MR610 (●, ○); (II) MR659 (■, □) and its *carD2* derivative MR664 (●, ○); (III) MR658 (■, □) and its *carD2* derivative MR663 (●, ○); (IV) MR410 (■, □) and its *carD1* derivative MR590 (●, ○).

standard strain. This is different from, although very closely related to, our standard strain DK1050 (see Materials and methods). Insertion Ω MR509::Tn5 was transduced into DK1622, and 200 Km^R transductants (all of which showed the expected Car^- phenotype) were tested for fruiting. None of them developed normally, indicating that the fruiting defect was attributable to a single mutation and that this was coincident with (or very tightly linked to) mutation *carD1*. Figure 4A illustrates the fruiting behavior of DK1622 and one of its *carD1* derivatives (MR563). Long after DK1622 had formed mature fruiting bodies, MR563 exhibited only weak signs of cell aggregation, as if it had been blocked at an early stage of development. All sib transductants displayed the same abnormal appearance on starvation plates as did MR563.

The disability of MR563 to develop normally was not a consequence of the lack of carotenoids. We separately transduced different Car^- mutations (*carB*, *carC*, or *carQ*) and Car^C mutations (*carA* or *carR*) into DK1622.

Figure 4. (A) Developmental behavior of *M. xanthus* wild-type strain (DK1622, left) and *carD1* mutant (MR563, right). Photographs were taken 4 days after the samples (10^9 cells/ml) were spotted on starviation plates. Bar, 2 mm.

(B) Influence of the cell density on the fruiting behavior of DK1622 (gray bars) and MR563 (black bars). Data are the number of fruiting bodies (ordinates) observed at different times (abscissas, in hr) on $10\text{-}\mu\text{l}$ samples of various cell densities that were spotted on starviation plates. The average of three or more replicas of each sample are represented. (I) 5×10^8 cells/ml; (II) 1.25×10^9 cells/ml; (III) 2.5×10^9 cells/ml; (IV) 5×10^9 cells/ml. (C) Phenotypic rescue of MR563 by the Fru^+ strain MR654. Cells from each strain were spotted on starviation plates, either alone or in a mixture. The number of spores of one or the other kind were counted after incubation for 6 days. Spores from MR654 (*carR3*) formed red colonies; those of MR563 formed yellow colonies.

From each transduction, several *Car* mutant colonies were tested, and they all formed normal fruiting bodies (not shown). The initiation of aggregation in *M. xanthus* depends on cell density (Kuspa et al. 1992). High cell density partially restored the ability of MR563 to form fruiting bodies. However, these were clearly outnumbered by the fruiting bodies formed by control samples of DK1622 (Fig. 4B).

Cell differentiation into myxospores was also affected by mutation *carD1*. The spores formed by MR563 on

starvation plates varied in different experiments $\sim 10^{-3}$ times the number of spores formed by DK1622. MR563 cells recovered their ability to sporulate on starvation plates when mixed with wild-type cells in a 1:1 ratio. Interestingly, the frequency of sporulation of the wild-type cells in the mixture with the *carD* mutant was lower than that of wild-type cells alone (Fig. 4C). The same effect has been described previously in *M. xanthus* mutants for the production of a factor, called A-factor, that acts as an intercellular signal during development (see below). Glycerol is known to induce the direct transformation of growing cells into myxospores (Dworkin and Gibson, 1964). Mutation *carD1* did not affect this aggregation-independent process. The addition of glycerol to exponentially growing cells produced the same number of spores in MR563 as in DK1622 (not shown).

Mutation *carD1* prevented the expression of several genes known to be activated specifically during development. To test this, we used 10 different *M. xanthus* strains, each of which has transposon Tn5-*lac* inserted at a distinct, developmentally activated gene, in the correct orientation for expression of the *lacZ* transcriptional probe. $\Omega\text{MR509::Tn5-132}$ was transduced into each of these strains, and the Km^R Tc^R derivatives were used for β -galactosidase expression analysis. As shown in Figure 5, *carD1* affected the developmental activation of seven of the genes tested. In some cases, *carD1* blocked activation of the gene completely (Tn5-*lac* insertions ΩDK4491 , ΩDK4521 , ΩDK4403 , ΩDK4401 , and ΩDK4435). In other cases, only a partial reduction in developmental activation of the gene was observed (ΩDK4514 and Ω4414).

In *M. xanthus*, several genes are known to be responsible for various intercellular signals (called A, B, C, and D factors) that control the morphological changes and the pattern of differential gene expression observed during fruiting body formation (Kaiser and Kroos 1993). We have found that gene *carD* lies far from all signaling genes for which a linked selectable marker was available. These were *asgA*, *asgB*, and *asgC* (A factor), *bsgA* (B factor), *csaA* (C factor), and *dsgA* (D factor). Insertion $\Omega\text{MR509::Tn5-132}$ (or $\Omega\text{MR509::Tn5}$) was transduced into *M. xanthus* strains carrying Tn5 (or Tn5-132) at a site linked to one of the signaling genes. Segregation for Km^R and Tc^R in these crosses showed no evidence of linkage of *carD1* to any of the tested loci (data not shown).

A short wild-type DNA fragment rescues the *Car* and *Fru* phenotype of the *carD* mutant

A 7.0-kb restriction fragment of wild-type DNA astride insertion site ΩMR509 was cloned in a plasmid vector (pDAH160; Km^R) that can be shuttled between *Escherichia coli* and *M. xanthus*. The chimeric plasmid was named pMAR503. Two other plasmids, carrying internal segments of the 7.0-kb DNA fragment, were named pMAR505 (3.3 kb) and pMAR513 (2.8 kb). These plasmids were transduced separately into the *carD::Tn5-132* (Tc^R) mutant by selecting for Km^R . As the plasmids

Figure 5. Effect of mutation *carD1* on the expression of developmentally activated genes. Each panel represents β -galactosidase production by a strain carrying Tn5-*lac* inserted at a different developmentally regulated gene (■, continuous lines), and by the corresponding *carD::Tn5-132* derivative (●, broken lines). Time zero indicates the time at which cell samples ($\sim 10^9$ cells/ml) were spotted on starvation plates. The strains are ordered according to the timing of β -galactosidase expression (see Fig. 8).

could not replicate in *M. xanthus* cells, transductants arose by integrative homologous recombination. In each case, the Car phenotype of many $\text{Km}^R \text{Tc}^R$ transductants was checked, and the three plasmids were found to complement the *carD1* mutation (Fig. 6). A few Car⁻ transductants found with pMAR503 should have originated by gene conversion (Stephens and Kaiser 1987). These results indicated that *carD1* was recessive to the corresponding wild-type allele and that the complete *carD* gene was likely to be contained in pMAR513.

For complementation analysis with portions of the 2.8-kb DNA fragment cloned in pMAR513, we used vector pSJM103. This contains the attachment site (*attP*) of a myxobacterial phage for the efficient integration at a separate locus in the *M. xanthus* chromosome (*attB*). Different portions of the 2.8-kb DNA fragment were

cloned in pSJM103 and transduced separately into the *carD* mutant. The smallest segment that rescued both the Fru and Car phenotype was a 1.2-kb *BssHII*-*NcoI* restriction fragment (Fig. 6). No segment was found that could rescue only the Car (or only the Fru) phenotype (not shown).

The expression of carD is regulated developmentally and unaffected by light

The cloning of *carD* allowed us to generate the *carD2* mutation mentioned above. This was done by cutting plasmid pMAR513 at the sole *SacI* site within the *M. xanthus* DNA (Fig. 6), followed by exonucleation of the single DNA tails and religation of the newly generated blunt ends. The new plasmid, which probably carries a 4-bp deletion within (or around) *carD*, was transduced into strain DK1622, by selecting for Km^R . All of the merodiploid transductants were Car⁺ Fru⁺, indicating that a new *carD* mutation, if present, was also recessive to the wild-type allele. Two Car⁺ Fru⁺ transductants were grown for 25 generations in the absence of kanamycin, and then plated for Car⁻ segregants. Several Car⁻ Km^S colonies were identified from the two cultures and all showed to be Fru⁻. They must have derived from intramolecular recombination events that resulted in the loss of the vector DNA and of the wild-type allele of *carD*. We concluded that the *SacI* site was within gene *carD* and that a new mutant allele (*carD2*) had been generated.

We examined the behavior of *carD2* in all experimental situations to which *carD1* had been subjected. In all cases, *carD2* showed exactly the same effects as *carD1*, with regard to the expression of either the light-activated or development-specific genes. Mutation *carD2* was also complemented by the 1.2-kb DNA fragment mentioned above (not shown).

The DNA clones were also used to create *lacZ* transcriptional fusions at the same *SacI* site where mutation *carD2* was generated. For this, the proximal *SmaI* sites at the two sides of *SacI* were used. The two *SmaI*-*SacI* fragments from pMAR503 (Fig. 6) were recloned separately into pDAH274 (Km^R), and the orientation of the fragments with respect to the *lacZ* probe of the vector was assigned by restriction analysis. Two chimeric plasmids, having *lacZ* inserted at the *SacI* site, each one in a different orientation, were transduced separately into *M. xanthus* strain DK1050. Selected transductants, originated by integrative homologous recombination, should carry a duplication of the cloned DNA and the *lacZ* probe inserted at the same site, and with the same orientation, as in the incoming plasmid. This is illustrated in Figure 7, which also shows the results of β -galactosidase expression analysis. In each case, data were obtained using a randomly picked colony from ≥ 50 sib transductants that had been checked previously for homogeneous behavior on X-gal plates. In addition to the copy of *carD* that was truncated by the *lacZ* probe, all of the transductants were expected to carry a wild-type al-

Figure 6. Restriction map and complementation ability of DNA fragments from *M. xanthus* strain DK1050. The thick lines represent the DNA fragments cloned in different plasmids (names at left, together with the size of the cloned DNA). Plasmids pMAR503, pMAR505, and pMAR513 derive from pDAH160 (Km^R) and integrate into the chromosome of *M. xanthus* by homologous recombination. Plasmids pMAR517 and pMAR518 derive from pSJM103 (Km^R) and integrate most of the times at a separate chromosomal site (*attB*) by site-specific recombination with the *attP* site of pSJM103 (see Materials and methods). Tn5 insertion site Ω MR509 is marked by an inverted triangle in pMAR505. The *SacI* (Sc) site used to generate mutation *carD2* is underlined. (Right) The Car phenotype of Km^R colo-

nies from the transduction of each plasmid into the *carD*::Tn5-132 mutant. The Fru phenotype of some randomly picked Car⁺ transductants carrying pMAR503, pMAR517, or pMAR518 is also indicated. Restriction sites: (A) *Apal*; (B) *Bam*HI; (Bs) *Bss*HI; (C) *Cla*I; (K) *Kpn*I; (N) *Nco*I; (S) *Sma*I; (Sa) *Sall*; (Sc) *Sac*I.

lele of that gene. In agreement with this assumption, they all showed a Car⁺ phenotype.

As seen in Figure 7, significant β -galactosidase activity was only detected in the strain with *lacZ* inserted in a particular orientation (drawn from left to right). Data of β -galactosidase expression in this strain, which should reveal the expression of gene *carD* itself, provided evidence for this gene being actively transcribed during the vegetative growth cycle in a light-independent manner. The *carD*::*lacZ* fusion was introduced into a *M. xanthus* strain that carried mutation *carA1*, and in different strains carrying lack-of-function mutations at *carQ*, *carR*, or *carS*. None of these mutations produced a significant change in the expression of *carD* in the dark or in the light (data not shown).

The *carD*::*lacZ* fusion at the *SacI* site was also transduced into the *M. xanthus* standard strain DK1622. As expected, all tested transductants showed a Car⁺ Fru⁺ phenotype. Cells of a randomly picked transductant were placed on starvation plates and β -galactosidase was assayed at different times during development. As shown in Figure 7, *carD* expression increased quickly after initiation of development and was shut off 5–6 hr later.

Discussion

The role of *carD* in the "light regulon" of *M. xanthus*

Previous studies have attempted to identify and characterize those genes involved in the carotenogenic response of the bacterium *M. xanthus* to blue light (*car* genes). Our present knowledge of these genes is summarized in the model depicted in Figure 1. This model takes into account the properties and interactions of different *car* mutations, the results of expression studies using transcriptional fusions to the *car* promoters, and the se-

quence analysis of various *car* regions (for review, see Hodgson and Murillo 1993).

Here, we describe the screening of 11,000 independent Tn5 insertions into the *M. xanthus* chromosome, during which 10 new carotenoid-less (Car⁻) mutants were isolated. Seven of these mutants have the transposon inserted at the previously known gene *carB*. This gene may contain a hot spot for integration of Tn5 (Berg et al. 1983). Another mutant carries Tn5 inserted at the previously known gene *carC*. The two other Car⁻ strains are affected at new *car* loci. This study deals with one of the new *car* loci, named *carD*.

As we and others have already reported, blue light strongly activates the promoters of the *carQRS* and *carBA* operons, and of gene *carC* (Balsalobre et al. 1987; Fontes et al. 1993; Hodgson 1993; Fig. 1). As deduced from expression analysis of the appropriate *lacZ* transcriptional fusions, all of these promoters are inactivated when *carD* is mutated (Fig. 2). This suggests that in the wild type, the *carD* gene product acts as a positive element at an early stage of the regulatory cascade initiated by the light stimulus.

In the model of Figure 1, it is not known how blue light inactivates the negative regulator CarR, or how this protein blocks the activity of CarQ. It has been proposed, however, that the sensitizer for the light action is protoporphyrin IX (Burchard and Hendricks 1969; McGowan et al. 1993). A role of *carD* in deactivation of CarR by light is dismissed, given the epistatic effect of *carD1* over a mutation at *carR*. Instead our data suggest that as proposed for CarQ, the *carD* gene product is involved directly in the light activation of the *carQRS* operon. Available data would fit various simple models on how CarQ and CarD interact to turn on the *carQRS* promoter. For example, one product could act as a transducer of the light signal to activate the other or both could be required to physically assemble at the promoter

Figure 7. Vegetative and developmental expression of gene *carD*. (Top) The site of insertion (*SacI*; Fig. 6) and the orientation of the probe in two *lacZ* fusions to *carD*.

(A) Time course of β -galactosidase (β -gal.) activity in dark-grown cultures of DK1050 derivatives having *lacZ* inserted from left to right (strain MR624; ●), or in the opposite orientation (strain MR625; ■). Stationary phase was reached at the time indicated by the arrow. (Inset) β -Galactosidase expression in MR624 growing exponentially in the dark (●) or the light (○). In stationary phase, no differences were observed between dark- and light-grown cultures of MR624 (not shown). (B) Developmental expression of β -galactosidase in the DK1622 derivative carrying the *carD::lacZ* fusion (strain MR665). At time zero, exponentially growing cells were spotted on starvation plates. At the indicated times, samples were taken for β -galactosidase analysis.

region. It is worth noting that in analyzing the amino acid sequence of CarQ, McGowan et al. (1993) did not find any of the peptide motifs known to bind DNA. Nor did they find homology between CarQ (or CarR) and the elements of the two-component systems that participate in the response to different environmental signals in various bacterial species (Parkinson and Kofoed 1992).

Mutation *carA1* causes constitutive transcription of the *carBA* operon and is epistatic over mutations at *carQ* (Martínez-Laborda and Murillo 1989). The epistasis of *carA1* also over the *carD1* mutation (Fig. 3) supports the particular arrangement of the *carD* and *carA* gene functions in the model of Figure 1. That *carD* controls the

carBA promoter only indirectly is well supported by the null effect of *carD2* on the expression of *carB* when the *carQRS* operon is transcribed from a constitutive promoter (Fig. 3). It is worth noting that the level of constitutive expression of *carQRS* is much higher when the *carD* mutation is present than when it is absent (Fig. 3I). One explanation might be that a factor normally binds at the 5' region of *carQRS*, thus hampering the run-through of the transcript driven by the constitutive promoter. The *carD* mutation would prevent that binding.

Constitutive expression of *carQRS* does not alter the light regulation of the *carC* promoter (Fig. 3II). Hence, this promoter is unlikely to be activated by the *carS* gene product in the same way as the *carB* promoter, a conclusion first suggested by other data. A point mutation at *carS* is known to generate a "superactive" CarS protein, as it causes high expression of *carB* in the dark. In addition, lack-of-function mutations at *carS* block the activity of the *carB* promoter (Balsalobre 1989; McGowan et al. 1993). None of these *carS* mutations affect the normal expression of *carC* (Fontes 1992). We have shown previously that genetic impairment of *carQ* does block light induction of the *carC* promoter (Fontes et al. 1993). It seems, then, that CarQ participates in turning on the *carC* promoter, but only after being activated by light, probably through deactivation of protein CarR. Light induction of *carC* also requires the normal operation of CarD, even when *carQRS* is expressed in a light-independent manner (Fig. 3II). Hence, CarD and CarQ also appear to interact with each other to activate the *carC* promoter.

The transcription of the *carD* gene occurs at a significant level during vegetative growth and is not regulated by light (Fig. 7). This is the first time that this kind of behavior has been recorded among the genes known to be involved in the light response of *M. xanthus*. Consistent with this, *carD* expression is not altered by mutations at genes (*carA*, *carQ*, *carR*, or *carS*) that regulate the activity of the light-inducible promoters (see Results). The post-transcriptional regulation of *carD* by light has not been investigated. Both mutations *carD1* (a Tn5 insertion) and *carD2* (a 4-base deletion) probably generate nonfunctional gene products. The growth rate of the two mutant strains looks normal; therefore, *carD* seems to be a dispensable gene. Continuous expression of *carD*, then, may assure a prompt reaction to the light stimulus or may serve other accessory functions.

The role of carD in the developmental program of M. xanthus

Mutation *carD1* blocks the fruiting body formation and sporulation that normally occur when *M. xanthus* cells are starved of nutrients (Fig. 4). A simple explanation for this could be that in the wild type, *carD* participates both in the response to light and in multicellular development. This would imply an unexpected link between the molecular mechanisms for these two processes. Tn5 mutations may have polar effects (Berg et al. 1980).

Hence, two distinct, linked genes could be involved in the double mutant phenotype of the *carD1* strain. Although this alternative cannot be ruled out completely, we consider it very unlikely for several reasons. A frame-shift mutation (*carD2*) near (upstream of) mutation *carD1* also results in a $\text{Car}^- \text{Fru}^-$ phenotype. Mutation *carD2* could affect more than one gene, particularly if it had damaged a promoter or a downstream gene were coupled translationally to *carD* (e.g., see Oppenheim and Yanofsky 1980; Crowell et al. 1987; Tomasiewicz and McHenry 1987). That *carD2* does not halt transcription is shown by the positive expression of a *lacZ* probe inserted at the same site where the mutation was generated (Fig. 7). This expression is activated transiently during development, which also favors the idea of a role of *carD* in that process. Finally, the 3' end of the shortest DNA fragment that rescues the double $\text{Car}^- \text{Fru}^-$ phenotype lies only 0.45 kb downstream of the *carD2* mutational site (Fig. 6).

Mutation *carD1* blocks fruiting because it prevents the activation of development-specific genes. The genes that we tested (Fig. 5) are part of a larger collection of genes identified by random mutagenesis with *Tn5-lac* and screening for development-specific expression of β -galactosidase (Kroos et al. 1986). *Tn5-lac* insertions in Figure 5 were selected from that collection to form a set of markers distributed uniformly throughout development (Fig. 8).

Developmental gene expression in *M. xanthus* is governed by various extracellular signals (Hagen et al. 1978; Kroos et al. 1986; Kuspa et al. 1986). The absence, by mutation, of a particular signal results in either lack or reduced expression of certain (*Tn5-lac*) markers. These are then said to exhibit absolute or partial dependence, respectively, on that signal. Four well-characterized signals, the A, B, C, and D factors, are required in various combinations to activate different markers (Kroos and Kaiser 1987; Kaiser and Kroos 1993; Fig. 8). Gene *carD* is distinct from the known signaling genes. These are three genes for factor A, *asgA*, *asgB*, and *asgC* (Kuspa and Kaiser 1989; Mayo and Kaiser 1989), and the genes *bsgA* (Gill and Cull 1986), *csgA* (Shimkets et al. 1983), and *dsgA* (Cheng and Kaiser 1989) for factors B, C, and D, respectively. A locus for a new intercellular signal (*esg*) has been identified recently. This is also distinct from *carD*. Contrary to *carD1*, *esg* mutations affect the expression of the *tps* gene [see below], and the restriction map of the *carD* locus differs from that of *esg* (Downard et al. 1993).

Comparison of our data with the signal dependence pattern of the *Tn5-lac* markers leads to various conclusions (see Fig. 8). *CarD* is unrelated to B- or D-type signaling. Mutation *carD1* does not affect expression of early markers ΩDK4455 ("partial B") and ΩDK4469 ("partial D-partial B"); however, it does block expression of early markers ΩDK4491 and ΩDK4521 , both of which show absolute dependence on factor A. A role for *CarD* in the production of the A factor itself is unlikely, as the *tps* gene ("absolute A") shows no *CarD* dependence. This points to the presence of two distinct A factor-dependent

Figure 8. Dependence of developmental markers on A, B, C, and D factors and on gene *carD*. [Representation taken from Kroos and Kaiser (1987).] The position of each marker along the x axis indicates the time at which its expression begins during development [for references, see Table 1; Kaiser and Kroos (1993) and references therein]. The pattern of dependence of each marker on intercellular factors can be read by following the horizontal and vertical lines to the lower corner of the diagram (see the text for an explanation on partial and absolute dependence). Markers showing absolute dependence on gene *carD* are enclosed in rectangular frames, whereas those showing partial dependence on gene *carD* are enclosed in elliptical frames (see Fig. 5).

mechanisms for gene activation, one of which requires the participation of *CarD*.

The *CarD*-dependence pattern of the other five markers matches their pattern of dependence on the C factor. "Partial-C" markers ΩDK4514 and ΩDK4414 exhibit partial dependence on *carD*, whereas "absolute-C" markers ΩDK4403 , ΩDK4401 , and ΩDK4435 , all exhibit absolute dependence on *carD* (Figs. 5 and 8). Thus, *carD* may be involved in the generation of the C factor, or it may participate in the C factor-mediated mechanism for gene activation. These two alternatives are not mutually exclusive, as C factor regulates its own expression positively (Kim and Kaiser 1991). The faulty production of an intercellular signal would explain the phenotypic rescue of the *carD1* mutant at high cell density or when mixed with wild-type cells (Fig. 4).

The effects of mutation *carD1* on A factor-dependent markers on the one hand, and on C factor-dependent markers on the other, are not interconnected. The C factor-dependent markers that we tested all exhibit abso-

lute dependence on A factor, but “partial C” markers Ω DK4514 and Ω DK4414 exhibit only partial dependence on *carD* (Fig. 8). CarD, then, seems to enter the developmental program separately at two distinct stages, one under the control of the A factor, and the other of the C factor. It should be noted that A- and C-type signaling have some features in common. In particular, they are both expressed only after the appropriate cell density has been reached (Kaiser and Kroos 1993).

Final remarks

The cellular processes in which *carD* participates, the induction of carotenoid synthesis and multicellular development, are both triggered by external stimuli (light or nutritional deprivation), and both entail the activation of specific genes. Carotenoid gene *carC* is regulated both by light and carbon starvation (Fontes et al. 1993), but no similar example of cross-regulation is known in the two sets of CarD-dependent genes described here (F.J. Nicolás, R.M. Ruiz-Vázquez, and F.J. Murillo, unpubl.). Thus, in *M. xanthus*, the actions of two different environmental signals converge in a common element (CarD) but diverge in the set of response genes. Specificity results from the interaction of CarD with other elements: CarQ for the light response, and factors A and C for the starvation response. More data should be gathered to understand better the action of CarD and to unravel its molecular “tricks”. Such data may provide important clues in understanding how myxobacteria sense their environment and how they react to it individually or as a group of cells.

Materials and methods

Bacterial strains, phages, and growth conditions

The *M. xanthus* strains used in this study are listed in Table 1, together with their phenotype, genotype, and origin. The standard strains DK1050 and DK1622 show normal light-induced synthesis of carotenoids (Car⁺) and starvation-induced fruiting body formation (Fru⁺). They both derive from the prototype *M. xanthus* strain FB (Kaiser 1979; Ruiz-Vázquez and Murillo 1984). Strains used in the analysis of the light response carried *car* mutations or *lacZ* fusions to *car* genes. The latter were either Tn5-*lac*-generated fusions (Kroos and Kaiser 1984) or in vitro-constructed gene fusions that were integrated into the *M. xanthus* chromosome by homologous recombination (see below). In both cases, the reporter gene retained the normal translation start signal preceded by stop codons in all three open reading frames; therefore, it produced transcriptional, but not translational fusions. Insertions of transposon Tn5 or Tn5-*lac* cause (Km^R), and insertion of Tn5-132 causes (Tc^R). Insertion sites are identified by Ω followed by letters and numbers (see Avery and Kaiser 1983).

Media and culture conditions were described previously by Balsalobre et al. (1987). The cells were illuminated exactly as described by Fontes et al. (1993). For generalized transduction between *M. xanthus* strains, phage Mx4-LA27 was used. The phage and the conditions used for transduction of Km^R and Tc^R were described by Avery and Kaiser (1983). Coliphage P1 *clr-100* cam was used to transfer plasmids from *E. coli* to *M. xanthus* (Gill et al. 1988).

Plasmids

Cloning vector pDAH160 (Hodgson 1993) carries a Km^R gene and the incompatibility region of phage P1 for transfer of the plasmid to *M. xanthus* by P1-specialized transduction. Those two elements are also present in all other plasmids used in this work. Plasmid pDAH274 (McGowan et al. 1993) carries a *lacZ* transcriptional probe. Plasmid pSJM103 (McGowan et al. 1993) contains *attP* of the myxobacterial phage Mx8. This promotes integration of the plasmid at high frequency by site-specific recombination into a site of the *M. xanthus* chromosome (*attB*), which is unlinked to any of the *car* loci.

Plasmid pDAH217 contains a *lacZ* transcriptional probe fused to the light-inducible promoter of the *carQRS* operon (see Fig. 1). As all other plasmids used in this study, pDAH217 cannot replicate in *M. xanthus* cells but can integrate into the *M. xanthus* chromosome by homologous recombination. Integration of pDAH217 produces a tandem duplication of the cloned DNA; therefore, a normal copy of the *carQRS* operon is generated (Hodgson 1993). Plasmid pDAH296 also carries a *lacZ* probe fused to the promoter region of *carQRS*. This plasmid, however, contains the IS50L element immediately upstream of the *carQRS* promoter; therefore the *lacZ* probe is expressed constitutively when the plasmid is integrated in the *M. xanthus* chromosome. Integration of pDAH296 also leaves a normal copy of the *carQRS* operon (Hodgson 1993). Plasmid pMAR206 contains a *lacZ* transcriptional probe fused to the light-inducible promoter of gene *carC* (Fontes et al. 1993).

DNA manipulation

The use of plasmid pDAH160 to clone wild-type DNA around a transposon insertion site has been described in detail previously (Hodgson 1993). We used this procedure to obtain plasmid pMAR503, which carries a 7-kb DNA fragment from the *M. xanthus* strain DK1050 (Fig. 6). This and all other plasmids constructed for this study were maintained in the *E. coli* strain MC1061 (Casadaban and Cohen 1980). Different portions of the DNA cloned in pMAR503 were recloned by using the appropriate restriction enzymes. All nucleic acid and enzymatic manipulations were done according to standard procedures (Maniatis et al. 1982). All enzymes came from Boehringer Mannheim.

Induction of fruiting body formation and sporulation

For induction of development, exponentially growing cells were washed and resuspended in TPM [10 mM Tris-HCl (pH 7.5), 1 mM KH₂PO₄, 8 mM MgSO₄], spotted on TPM agar (1.5%) plates, and incubated at 33°C. The fruiting bodies formed after different incubation times were counted under a Zeiss dissecting microscope. For counting of spores, the plates were incubated for 6 days and the same number of spots from each sample were scraped from the agar and resuspended in TPM. The spores were counted as temperature- and sonication-resistant colony-forming units (Kuspa and Kaiser 1989). Induction of sporulation in exponentially growing cultures was done by the addition of 0.5 M glycerol, as described by Dworkin and Gibson (1964).

Expression of β -galactosidase

Rapid determination of β -galactosidase production was carried out by examining colony color on plates containing 40 μ g/ml of X-gal. Quantitative analysis of β -galactosidase on dark- or light-grown liquid cultures was performed as described previously by Balsalobre et al. (1987). Induction by light of the *carC* promoter

Table 1. *Myxococcus xanthus* strains used in this work

Strain	Phenotype ^a	Genotype ^a	Source or derivation ^b
I.			
DK1050	Car ⁺ Fru ⁺		Ruiz-Vázquez et al. (1984)
DK1622	Car ⁺ Fru ⁺		Kaiser (1979)
MR654	Car ^C Fru ⁺ Km ^R	<i>carR3</i> , Ω DK1611::Tn5	F.J. Nicolás (this study)
II.			
DK406	Car ^C	<i>carR4</i>	Balsalobre et al. (1987)
MR7	Car ^C	<i>carA1</i>	Balsalobre et al. (1987)
MR144	Car ⁺ Tc ^R	Ω DK1910::Tn5-132	Martínez-Laborda et al. (1986)
MR151	Car ^C	<i>carR3</i>	Balsalobre et al. (1987)
MR397	Lac ^I Km ^R	<i>carQ'::lacZ</i>	pDAH217 $\times$ DK1050 $\rightarrow$ Km ^R
MR398	Lac ^C Km ^R	<i>carR4</i> , <i>carQ'::lacZ</i>	pDAH217 $\times$ DK406 $\rightarrow$ Km ^R
MR401	Car ⁻ LacZ ^I Km ^R	<i>carB::Tn5-lac</i>	Balsalobre et al. (1987)
MR406	Lac ^C Km ^R	<i>carR3</i> , <i>carB::Tn5-lac</i>	Balsalobre et al. (1987)
MR410	Lac ^C Km ^R	<i>carA1</i> , <i>carB::Tn5-lac</i>	Balsalobre et al. (1987)
MR418	Car ⁻ Lac ^I Tc ^R	<i>carB::Tn5-lac-132</i>	Martínez-Laborda et al. (1990)
MR461	Car ⁻ Tc ^R	<i>carC::Tn5-132</i>	Balsalobre et al. (1987)
MR553	Lac ^I Km ^R	<i>carC'::lacZ</i>	Fontes et al. (1993)
MR555	Lac ^C Km ^R	<i>carR3</i> , <i>carC'::lacZ</i>	Fontes et al. (1993)
MR609	Lac ^C Km ^R	IS50L- <i>carQ'::lacZ</i>	pDAH296 $\times$ DK1050 $\rightarrow$ Km ^R
MR655	Lac ^I Km ^R	<i>carB::Tn5-lac</i>	MR401 $\times$ DK1622 $\rightarrow$ Km ^R
MR658	Lac ^C Km ^R Tc ^R	<i>carB::Tn5-lac</i> , Ω DK1910::Tn5-132	MR144 $\times$ MR655 $\rightarrow$ Tc ^R
MR656	Lac ^I Km ^R	<i>carC'::lacZ</i>	pMAR206 $\times$ DK1622 $\rightarrow$ Km ^R
MR659	Lac ^I Km ^R Tc ^R	<i>carC'::lacZ</i> , Ω DK1910::Tn5	MR144 $\times$ MR656 $\rightarrow$ Tc ^R
III.^c			
DK2630	Km ^R	Ω DK1519::Tn5	Shimkets and Kaiser (1982)
DK3261	Km ^R	Ω DK1867::Tn5	Cheng and Kaiser (1989)
DK4746	Km ^R	Ω DK4678::Tn5	Mayo and Kaiser (1989)
DK5057	Km ^R	Ω DK4560::Tn5	Kuspa and Kaiser (1989)
DK5061	Km ^R	Ω DK4561::Tn5	Kuspa and Kaiser (1989)
DK5209	Tc ^R	<i>bsgA330</i>	Gill and Cull (1986)
IV.			
DK4290	Lac ^D	<i>tps(Δ4273)::Tn5-lac</i>	Kroos and Kaiser (1987)
DK4293	Lac ^D	Ω DK4401::Tn5- <i>lac</i>	Kroos et al. (1986)
DK4368	Lac ^D	Ω DK4403::Tn5- <i>lac</i>	Kroos et al. (1986)
DK4469	Lac ^D	Ω DK4469::Tn5- <i>lac</i>	Kroos et al. (1986)
DK4491	Lac ^D	Ω DK4491::Tn5- <i>lac</i>	Kroos et al. (1986)
DK4514	Lac ^D	Ω DK4514::Tn5- <i>lac</i>	Kroos et al. (1986)
DK4521	Lac ^D	Ω DK4521::Tn5- <i>lac</i>	Kroos et al. (1986)
DK5204	Lac ^D	Ω DK4435::Tn5- <i>lac</i>	Kroos et al. (1986)
DK5206	Lac ^D	Ω DK4455::Tn5- <i>lac</i>	Kroos et al. (1986)
DK5279	Lac ^D	Ω DK4414::Tn5- <i>lac</i>	Kroos and Kaiser (1987)

Strains are classified in different groups for clarity: (I) Standards; (II) strains used in the study of the response to light; (III) strains for the analysis of linkage to signaling genes; (IV) strains carrying developmentally regulated Tn5-*lac* insertions. Strain MR654, a DK1622 derivative, is included in group I because it shows a normal Fru⁺ phenotype and was used here only for the phenotypic complementation of a Fru⁻ mutant (Fig. 4). Other strains are introduced in the text.

^aOnly the phenotypes and genotypes relevant to this work are indicated. (Lac^I) Light-inducible synthesis of β -galactosidase; (Lac^C) high level of β -galactosidase in the dark and in the light; (Lac^D) developmentally induced synthesis of β -galactosidase. All strains in group II derive from DK1050, unless indicated otherwise in the Source or derivation column. All strains in groups III and IV derive from DK1622.

^bA brief description is given of the way some strains were constructed for this work. In these cases, the plasmids or strains used as donor of the antibiotic resistance marker are named in the first place.

^cEach strain in this group carries Tn5 inserted at a site linked to a distinct signaling gene. The exception is DK5209, which contains the Tc^R-encoding *Bgl*III fragment from Tn10 inserted in the *bsgA* gene (formerly *ssbA*).

requires that the cells enter the stationary phase (Fontes et al. 1993). β -Galactosidase expression in strains carrying a *carC'::lacZ* fusion was induced in cultures at the late exponential phase of growth. Quantitative analysis of β -galactosidase in developing cells was carried out as described by Kroos et al. (1986). Only the period of sonication on glass beads was modified (for

maximum spore breakage), as a different apparatus was used (Braun Labsonic U). Reported data of enzyme-specific activities, given in nanomoles of *o*-nitrophenol produced/min per milligram of protein are the average of three or more independent determinations. Standard deviations never exceed 10% of the average.

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