

## Differential Expression of the Three Multicopper Oxidases from *Myxococcus xanthus*<sup>∇</sup>

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Received 2 March 2007/Accepted 24 April 2007

*Myxococcus xanthus* is a soil bacterium that undergoes a unique life cycle among the prokaryotes upon starvation, which includes the formation of macroscopic structures, the fruiting bodies, and the differentiation of vegetative rods into coccoid myxospores. This peculiarity offers the opportunity to study the copper response in this bacterium in two different stages. In fact, *M. xanthus* vegetative rods exhibit 15-fold-greater resistance against copper than developing cells. However, cells preadapted to this metal reach the same levels of resistance during both stages. Analysis of the *M. xanthus* genome reveals that many of the genes involved in copper resistance are redundant, three of which encode proteins of the multicopper oxidase family (MCO). Each MCO gene exhibits a different expression profile in response to external copper addition. Promoters of *cuoA* and *cuoB* respond to Cu(II) ions during growth and development; however, they show a 10-fold-increased copper sensitivity during development. The promoter of *cuoC* shows copper-independent induction upon starvation, but it is copper up-regulated during growth. Phenotypic analyses of deletion mutants reveal that CuoB is involved in the primary copper-adaptive response; CuoA and CuoC are necessary for the maintenance of copper tolerance; and CuoC is required for normal development. These roles seem to be carried out through cuprous oxidase activity.

Like other myxobacteria, *Myxococcus xanthus* bacteria glide on the soil either as single cells or more typically as groups feeding cooperatively. However, under conditions of nutrient depletion, this bacterium undergoes a developmental program, unique among the prokaryotes, in which cells come together in a coordinated way to produce multicellular fruiting bodies within which they sporulate, converting the vegetative cells into myxospores. During this differentiation process, long vegetative rods reshape and become coccoids. This aggregation and differentiation of bacteria requires a complex interaction of signals controlling gene expression in time and space (22). This ability has converted this deltaproteobacterium in a model for studying prokaryotic morphogenesis and differentiation.

*M. xanthus* is common in soils rich in organic materials and rotting wood. In these niches, this bacterium has to frequently cope with toxic concentrations of copper ions, since levels of this metal in noncontaminated soils fluctuate between 2 and 100 mg/kg. This means that the *M. xanthus* life cycle under normal environmental circumstances is accomplished in the presence of relatively high concentrations of this transition metal. Copper is an essential trace element, but an excess is toxic. The importance of maintaining copper homeostasis is further reinforced by the presence in the cells of several families of proteins which are known to control the intracellular concentration of metal ions or that help confine them to vital roles (for reviews, see references 10, 29, 33, and 37). Moreover,

in higher-order organisms, such as humans, disturbed copper homeostasis has been implicated in diseases such as Menkes and Wilson syndrome, Alzheimer's disease, Parkinson's disease, and cystic fibrosis (5).

The copper response in *M. xanthus* seems to be more complex than in other bacteria. It has been reported that copper induces the accumulation of carotenoids in dark-grown cultures of *M. xanthus*, activating the transcription of the structural genes for carotenoid synthesis (27). This work has also shown that copper induces other unknown cellular mechanisms that confer tolerance to this metal during vegetative growth. It remains unknown which other mechanisms are responsible for this Cu-regulated adaptive response. The *M. xanthus* genome was recently sequenced (12), and analysis of this genome by our group has revealed a plethora of gene products with sequence similarities to proteins known to be involved in copper handling and trafficking in other organisms, most of which are redundant. This finding is indicative that copper homeostasis mechanisms in this bacterium could be different during growth and development. Among others, we have identified genes that encode three copper-translocating P-type ATPases, three Cus systems, and three multicopper oxidases (MCOs).

First, we concentrated our attention on three paralogous genes that encode MCOs, which have been named *cuoA*, *cuoB*, and *cuoC* (for cuprous oxidases). The MCO family is defined by the presence of three spectroscopically different copper centers. Enzymes that belong to this family include laccases, ascorbate oxidases, and ceruloplasmin in eukaryotes. Even though several bacterial MCOs have also been described, the biological roles of MCOs of prokaryotic origins are diverse and

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<sup>∇</sup> Published ahead of print on 4 May 2007.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description <sup>a</sup>	Reference
<b>Strains</b>		
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZΔM15]</i>	40
<i>M. xanthus</i>		
DZF1	<i>pilQ1</i>	28
JM51AIF	<i>ΔcuoA Gal<sup>r</sup> Km<sup>s</sup></i>	This study
JM51AZY	<i>cuoA-lacZ Km<sup>r</sup></i>	This study
JM51ADWZY	<i>cuoA-lacZ Km<sup>r</sup></i>	This study
JM51BIF	<i>ΔcuoB Gal<sup>r</sup> Km<sup>s</sup></i>	This study
JM51BZY	<i>cuoB-lacZ, Km<sup>r</sup></i>	This study
JM51CIF	<i>ΔcuoC Gal<sup>r</sup> Km<sup>s</sup></i>	This study
JM51CDWZY	<i>cuoC-lacZ Km<sup>r</sup></i>	This study
<b>Plasmids</b>		
pBJ113	<i>Km<sup>r</sup> galK</i>	21
pKY481	<i>lacZ Km<sup>r</sup></i>	7
pKY481-CuoA	<i>cuoA-lacZ Km<sup>r</sup></i>	This study
pKY481-FICuoA	<i>cuoA-lacZ Km<sup>r</sup></i>	This study
pKY481-CuoB	<i>cuoB-lacZ Km<sup>r</sup></i>	This study
pKY481-CuoCDW	<i>cuoC-lacZ Km<sup>r</sup></i>	This study
pAMDcuoA	<i>ΔcuoA Km<sup>r</sup></i>	This study
pAMDcuoB	<i>ΔcuoB Km<sup>r</sup></i>	This study
pAMDcuoC	<i>ΔcuoC Km<sup>r</sup></i>	This study

<sup>a</sup> Gal<sup>r</sup> means that the strain is able to grow in the presence of galactose.

apparently unrelated (9, 11, 15, 17, 19, 20, 35, 36). Some of them have been implicated in copper tolerance (3, 6, 23, 31). Nevertheless, in those bacteria where two MCOs have been involved in copper homeostasis, only one gene is chromosomally encoded, while the other is plasmid borne (10, 33, 38). The coexistence in the *M. xanthus* genome of three paralogs for MCOs raises intriguing questions about their physiological roles in copper homeostasis along the life cycle.

To clarify possible alternatives, we first studied how growth

and development alter the Cu-adaptive response in *M. xanthus*. Second, after characterization of the three MCO genes, we considered the effect of copper and the life cycle on the expression of each promoter. The correlation between the copper phenotypes of the three mutants during each developmental stage with differential expression of the MCO genes indicates that *M. xanthus* needs the coordinate action of the three proteins to complete a life cycle in the presence of elevated levels of copper ions.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *M. xanthus* and *Escherichia coli* strains used in this study are summarized in Table 1, together with their genotypes and origins. *M. xanthus* strains were grown with shaking at 30°C in liquid-rich Casitone-Tris (CTT) medium (18); CTT agar plates contained 1.5% Bacto agar (Difco). When necessary, kanamycin (40 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (100 μg/ml), or galactose (10 mg/ml) was added. *E. coli* strains were grown at 37°C in Luria-Bertani medium, which was supplemented with ampicillin (50 μg/ml), kanamycin (25 μg/ml), or X-Gal (25 μg/ml) when needed. Specific growth conditions are described in the corresponding Results section.

In metal tolerance experiments, the ranges of metals assayed (in mM) were as follows: nickel, 0.1, 0.5, 1, 2, 3, 4, 5, and 6; zinc, 0.05, 0.1, 0.25, 0.5, and 1; cobalt, 0.05, 0.25, 0.5, 1, 2, 3, 4, and 5; cadmium, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.5, and 0.6; silver, 0.02, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, and 0.5; ferrous ions, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8; and ferric ions, 0.1, 0.5, 1, 2, and 3. Copper was tested as Cu<sup>2+</sup> by adding 0.02, 0.04, 0.06, 0.08, 0.1, 0.25, 0.5, 1, or 2 mM of copper sulfate. To test Cu<sup>+</sup>, an excess of ascorbate (1 mM) was added to the medium plus the concentrations of Cu<sup>2+</sup> indicated above.

**Developmental conditions, spore quantification, and germination.** Clone fruiting (CF) medium (16) was used to induce development. Developmental conditions used were the same as those previously reported (25). When required, metals were added at the concentrations indicated in the figures. Specific developmental conditions are described in Results. For counting spores, 10 drops of 20 μl each were spotted on a 10-cm petri dish containing CF medium with various copper sulfate concentrations. At different times, the fruiting bodies of one plate were harvested and resuspended in 200 μl of TM buffer (10 mM Tris-HCl [pH 7.6], 1 mM MgSO<sub>4</sub>). Fruiting bodies were dispersed and rod-shaped cells disrupted by sonication. Myxospores were counted in a Petroff-Hausser chamber. For germination, sonicated samples obtained at 72 h of starvation were heated at 50°C for 2 h and then diluted and inoculated on CTT agar plates for 6 to 7 days to obtain colonies.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Purpose	Sequence (5'→3') <sup>a</sup>
lcsABamA	Amplification of upstream of <i>cuoA</i> (pKY481-CuoA)	<u>TTGGGATCCATGAAGCCTCTTCACGAATG</u>
lcsAKpn	Amplification of upstream of <i>cuoA</i> (pKY481-CuoA)	<u>CAGGGTACCGGCCATGAACGGCACTTCAC</u>
lcsBam2	Amplification of upstream of <i>cuoA</i> (pAMDcuoA)	<u>CAGGGATCCCAGTGCATGAAGCCTCTTCACG</u>
lacEco	Amplification of upstream of <i>cuoA</i> (pAMDcuoA)	<u>GTGGAATTCATGAACGGCACTTCACGGCTC</u>
lacPst	Amplification of downstream of <i>cuoA</i> (pAMDcuoA)	<u>CGGCTGCAGGGCCTCCAGCAGTGAACCCG</u>
lacBam2	Amplification of downstream of <i>cuoA</i> (pAMDcuoA)	<u>TGGGGATCCATGCGGAGGGTTCACG</u>
lcsAXhoF	Amplification of upstream of <i>cuoA</i> (pKY481-FICuoA)	<u>GCCTCGAGCCGAGGTACACGCGCCTCGGG</u>
lcsABamR	Amplification of upstream of <i>cuoA</i> (pKY481-FICuoA)	<u>CGGGATCCATCCGCAGTCGCACCCGCTC</u>
lcsBBamA	Amplification of upstream of <i>cuoB</i> (pKY481-CuoB)	<u>CTGGGATCCATGGTGGTGTGTCTCTCA</u>
lcsBKpn	Amplification of upstream of <i>cuoB</i> (pKY481-CuoB)	<u>CGCGGTACCCTGGTCCGAAGCCGAGAAC</u>
lcsBBam2	Amplification of upstream of <i>cuoB</i> (pAMDcuoB)	<u>CTGGGATCCATGGTGGTGTGTCTCTC</u>
lcsBXba	Amplification of upstream of <i>cuoB</i> (pAMDcuoB)	<u>GGTCTAGAGCACGGCGGTGGAGCTGGAG</u>
lcsBEco	Amplification of downstream of <i>cuoB</i> (pAMDcuoB)	<u>CCCGAATTCCTCGGGCTCCTGCCGGCTTG</u>
lcsBam	Amplification of downstream of <i>cuoB</i> (pAMDcuoB)	<u>GAGGATCCCAATCCGGACGTGCGCTTTG</u>
lcsCB2	Amplification of upstream of <i>cuoC</i> (pAMDcuoC); amplification of upstream of <i>cuoA</i> (pKY481-CuoCDW)	<u>TGGGATCCATGCTCCGCCGCGTCAGCG</u>
lcsCK	Amplification of upstream of <i>cuoC</i> (pAMDcuoC); amplification of upstream of <i>cuoA</i> (pKY481-CuoCDW)	<u>CTGGTACCTTCGGGCGCGTGGACGTGG</u>
lcsCB	Amplification of downstream of <i>cuoC</i> (pAMDcuoC)	<u>GTGGATCCACCTCGGACGAGCTGCGC</u>
lcsCE	Amplification of downstream of <i>cuoC</i> (pAMDcuoC)	<u>CTGAATTCCTGCTCCCCCTTCCCCCTGGC</u>

<sup>a</sup> Restriction sites added to the sequences are underlined.

**Construction of in-frame deletion strains.** Plasmids pAM $\Delta$ cuoA, pAM $\Delta$ cuoB, and pAM $\Delta$ cuoC were constructed for in-frame deletion of the *cuoA*, *cuoB*, and *cuoC* genes, respectively. Two fragments, upstream and downstream of each gene, were PCR amplified using the appropriate pairs of primers specified in Table 2. The amplified products were then digested and cloned into pBJ113, also digested with compatible enzymes. The resulting plasmids carrying the corresponding gene deletion were introduced into wild-type (WT) *M. xanthus* by electroporation. Several randomly chosen Km<sup>r</sup> merodiploids (*cuo*<sup>+</sup>) were analyzed by Southern blot hybridization for the proper recombination event. One positive strain was then grown in the absence of kanamycin and plated on CTT agar plates containing galactose. Southern blot analysis was again used to analyze positives and select a galactose-resistant (Gal<sup>r</sup>) Km<sup>s</sup> strain with in-frame deletion for each gene. The strains thus obtained have been designated JM51AIF, JM51BIF, and JM51CIF, containing deletions in *cuoA*, *cuoB*, and *cuoC*, respectively.

**Construction of strains harboring translational *lacZ* fusions and gene reporter studies.** Appropriate oligonucleotide pairs (Table 2) were used to amplify the fragments encompassing the upstream regions of the corresponding genes. A BamHI site in each primer was introduced either at the start codon or inside the genes and in-frame with the BamHI site existing in the *lacZ* gene of plasmid pKY481. PCR products were digested and ligated to pKY481, which was previously linearized by digestion with compatible enzymes for each PCR product. The resulting plasmids were introduced into *M. xanthus* WT by electroporation. Kanamycin-resistant colonies were analyzed as previously reported (4). All of the strains thus constructed harbor a WT copy of the three MCO genes. Strains containing fusions were incubated at 30°C on CTT and CF agar plates containing different copper concentrations. Cell extracts were obtained at various times by sonication and analyzed for  $\beta$ -galactosidase as previously reported (26). The protein content was determined by using the Bio-Rad protein assay. Specific activities are expressed as nmol of *o*-nitrophenol produced per minute and mg of protein.

**Transmission and scanning electron microscopy.** For transmission electron microscopy, cells were plated on CTT or CF agar plates to an optical density at 600 nm (OD<sub>600</sub>) of 15. Cells were treated as previously described (26). Photographs were taken in a Zeiss TEM902 transmission electron microscope (TEM) at 80 kV. For scanning electron microscopy, cells and fruiting bodies were fixed with glutaraldehyde vapors for 24 h at room temperature. They were then postfixed with osmium tetroxide under the same conditions described above. Dehydration was accomplished by a graded series of ethanol. Samples were then critical-point dried and sputter coated with gold. Photographs were taken in a Zeiss DSM950 scanning electron microscope (SEM).

**MCO activity determinations.** Cell extracts were obtained by sonication as described above for  $\beta$ -galactosidase activity determinations. Cells were harvested at 24 h of incubation in the absence or presence of 300 or 600  $\mu$ M copper. Assays were performed utilizing the following aromatic compounds as substrates under the conditions reported: 3,3-dimethoxybenzidine (30); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 4-hydroxy-3,5-dimethoxybenzaldehyde (24); and 2,6-dimethoxyphenol (14).

Cuprous oxidase activities were measured in terms of rates of oxygen consumption by using an oxygraph instrument (Hansatech) at 37°C as described by Singh et al. (35). Initial rates were calculated from the recorded curves of oxygen consumption versus time. The substrate, Cu(I), was added as a complex, [Cu(I)(MeCN)<sub>5</sub>]PF<sub>6</sub>, which releases free Cu(I) in solution. Stock solutions of Cu(I) were freshly prepared in degassed and argon-purged acetonitrile and subsequently diluted anaerobically by using gas-tight syringes. Reactions were initiated by addition of the substrate (0.5 mM) to an air-saturated mixture containing cell extract, 100 mM sodium acetate buffer, pH 5, and 5% acetonitrile. Control assays lacking the cell extracts or the substrate were also carried out. Specific activity is expressed as nmol of Cu(I) oxidized per minute per mg of protein, and activities are mean values from six assays with crude extracts prepared from cells derived from two independent cultures.

**Nucleotide sequence accession numbers.** The gene identifier for *cuoA* is MXAN\_3420, accession number ABF91184; for *cuoB*, MXAN\_3425, accession number ABF86571; and for *cuoC*, MXAN\_3432, accession number ABF89931.

## RESULTS

**Effect of copper on *M. xanthus* and Cu-regulated adaptive response during growth and development.** We have studied *M. xanthus* copper tolerance during growth and development under two different conditions. In the first set of experiments,

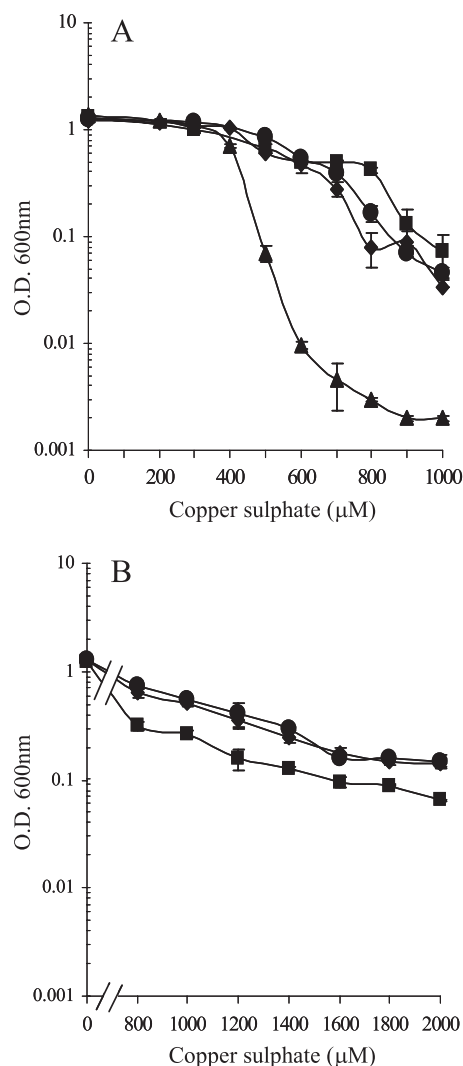


FIG. 1. Effect of copper on *M. xanthus* growth. The WT strain is represented by circles, the  $\Delta$ cuoA mutant by squares, the  $\Delta$ cuoB mutant by triangles, and the  $\Delta$ cuoC mutant by diamonds. (A) Copper tolerance of nonpreadapted cells to copper. Cells grown in the absence of the metal were diluted to an OD<sub>600</sub> of 0.05 in fresh CTT liquid media containing the indicated copper sulfate concentrations. The OD<sub>600</sub> indicated in the figure was monitored after 24 h of incubation. (B) Copper tolerance of Cu-preadapted cells. The WT strain and  $\Delta$ cuoA and  $\Delta$ cuoC mutants were grown in the presence of 600  $\mu$ M copper sulfate prior to dilution into fresh CTT liquid media containing different copper sulfate concentrations. As above, the OD<sub>600</sub> was determined at 24 h of incubation. Error bars indicate standard deviations.

cells were grown in the absence of external copper. For vegetative growth studies, these cells were diluted into fresh CTT liquid media supplemented with different copper concentrations and incubated for 24 h at 30°C. As can be observed in Fig. 1A, WT cells grew with copper concentrations up to 400  $\mu$ M at rates similar to those observed in the absence of metal. At higher concentrations, growth yields were lower, and no growth was observed at copper concentrations of 1,000  $\mu$ M. The difference in growth was the result of an increase in the generation time but not in the length of the lag phase (data not shown).

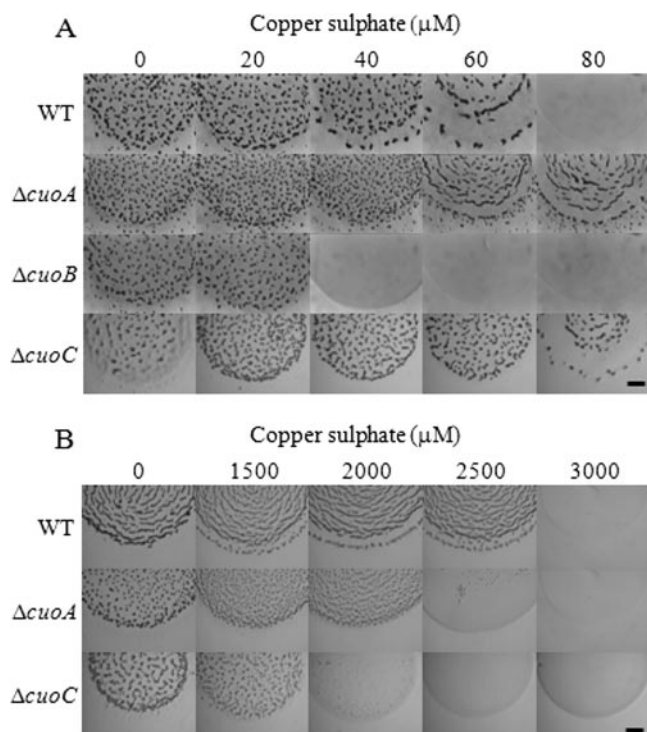


FIG. 2. Effect of copper on fruiting body formation. (A) Cells incubated in the absence of copper were concentrated at an OD<sub>600</sub> of 15 and spotted on CF medium with the copper sulfate concentrations indicated in each photograph. (B) Cells grown in the presence of 600 μM copper sulfate were spotted on CF agar plates with the metal concentrations illustrated. Photographs were taken at 72 h of incubation. Bar, 1 mm.

To study *M. xanthus* copper sensitivity during development, cells grown in the absence of copper were centrifuged and concentrated in TM buffer, and 10-μl drops were then spotted on starvation CF agar (where cells develop) containing different copper concentrations. As shown in Fig. 2A, WT cells aggregated only with copper concentrations up to 60 μM. At higher concentrations, cells died. We have also analyzed the sporulation and germination efficiencies of this bacterium in the presence of copper, and we have found that the myxospore yield was decreasing as the copper concentration was increasing (Fig. 3A, black bars). Copper addition also reduced the percentage of myxospores that were able to germinate (Fig. 3B, black bars).

The copper tolerance ratio (tolerance of growing cells versus tolerance of developing cells) was around 17. This metal tolerance ratio was much higher for copper than for other metals, such as nickel, zinc, cobalt, cadmium, silver, ferrous, and ferric ions (Table 3). The addition of an excess of ascorbate to the medium containing Cu<sup>2+</sup>, to promote the reduction of the metal to Cu<sup>+</sup>, increased the copper sensitivity only of developing cells (Table 3).

Since we recently demonstrated that *M. xanthus* copper resistance mechanisms are inducible (27), in a second set of experiments we tested the tolerance of cells during growth and development when they were preincubated in the presence of a copper concentration high enough to induce all of the mech-

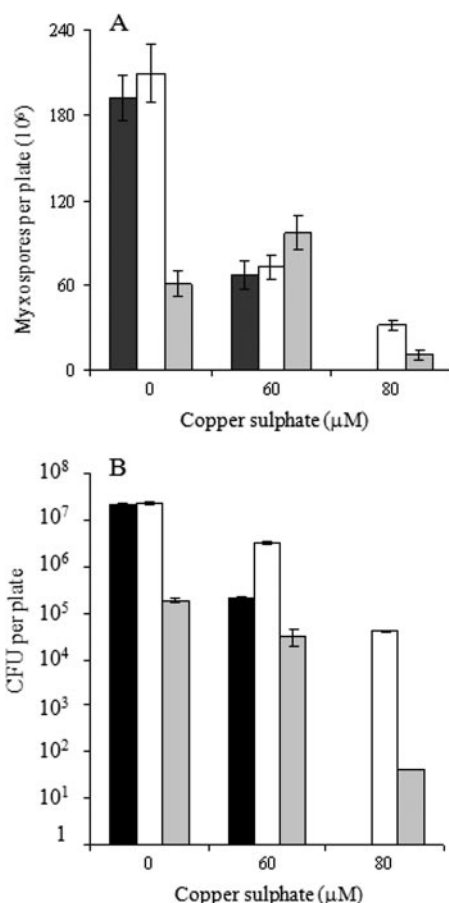


FIG. 3. Quantification of spore yield (A) and germination of the myxospores (B) for the WT strain (black bars) or the *ΔcuoA* (white bars) or *ΔcuoC* (gray bars) mutant. Error bars, standard deviations.

TABLE 3. *M. xanthus* tolerance of different metals during growth and development<sup>a</sup>

Metal	Maximum concn of metal (mM) allowing viability in medium		TR (CTT/CF) <sup>b</sup>
	CTT	CF	
Cu <sup>2+</sup>	1.0	0.06	17.0
Cu <sup>+</sup> <sup>c</sup>	1.0	0.04	25.0
Ni <sup>2+</sup>	6.0	4.0	1.5
Zn <sup>2+</sup>	0.5	0.5	1.0
Co <sup>2+</sup>	1.0	4.0	0.25
Cd <sup>2+</sup>	0.3	0.1	3.0
Ag <sup>+</sup>	0.25	0.05	5.0
Fe <sup>2+</sup>	7.0	7.0	1.0
Fe <sup>3+</sup>	2.0	1.0	2.0

<sup>a</sup> Cells grown in the absence of copper were concentrated to an OD<sub>600</sub> of 15, and 10 μl was spotted on CTT (for growth) or CF (for development) agar plates with different metal concentrations. Values shown represent the highest metal concentrations tested where cell viability was detected. Viability was determined by taking cells from the media with metals and streaking them on CTT agar plates with no copper. These plates were incubated for 1 week at 30°C to get colonies.

<sup>b</sup> The tolerance ratio (TR) was calculated as the quotient between the maximal metal concentrations where viability was observed.

<sup>c</sup> Cu<sup>+</sup> was obtained by the addition of Cu<sup>2+</sup> and 1 mM ascorbate. The concentrations indicated correspond to those of Cu<sup>2+</sup> added to the medium.



organisms involved in resistance to this metal. In this case, we performed the same type of experiments reported above but with cells previously grown in CTT medium containing 600  $\mu\text{M}$  copper sulfate. Preadapted cells were able to grow with up to 2,000  $\mu\text{M}$  copper, but generation times lengthened considerably as copper concentrations were increased (Fig. 1B). However, it is noteworthy that during development, preadapted cells aggregated even with copper concentrations as high as 2,500  $\mu\text{M}$  (Fig. 2B).

This elevated copper tolerance of preadapted cells during development versus that during growth could be attributed to the fact that experiments were carried out in different experimental settings. In the case of vegetative growth, cells were diluted to an  $\text{OD}_{600}$  of 0.05 and incubated in liquid medium with shaking, whereas they were concentrated to an  $\text{OD}_{600}$  of 15 before being plated on CF agar plates to study development. It is known that *M. xanthus* is a social bacterium that can face unfavorable environmental changes more efficiently when it is forming a community than when it is isolated. Therefore, we decided to test copper sensitivities of copper-preincubated and nonpreincubated cultures on CTT agar plates after concentrating cells to an  $\text{OD}_{600}$  of 15. In this case, we observed that cultures exhibited the same copper sensitivity on CTT agar plates as that reported above for liquid medium (data not shown).

These results clearly indicate that the copper preincubation step amplified the *M. xanthus* copper response only 2.5-fold in growing cells but as much as 41.7-fold in developing cells, indicating that development is the critical step for copper toxicity.

**MCOs in *M. xanthus* genome.** To study the *M. xanthus* copper response at the genetic level, we searched the genome for genes that have been involved in copper resistance and homeostasis in other bacteria, and we found that most of them are redundant. We concentrated our attention on *M. xanthus* MCOs, named CuoA, CuoB, and CuoC. We defined as the start codon for *cuoA* the first ATG found downstream of a termination codon in the same reading frame. This start codon exhibits a putative ribosome-binding site (AGAGG) 5 bp upstream. The coding sequence starting at this codon encodes a protein of 481 amino acids, where 80% of the triplets use either C or G as the third base, a codon usage typical of *M. xanthus* genes. The protein encoded by this gene exhibits a signal sequence at the N-terminal end, which indicates that it must be secreted to the periplasmic space by the Sec system.

In the case of *cuoB*, an ATG triplet with a putative ribosome-binding site (AGAGG) 10 bp upstream has been chosen as the start codon. The protein encoded by this gene contains 440 amino acids, with a codon usage where 88% of the triplets contain G or C as the third base. CuoB shows a signature in the N-terminal portion (RRQFI followed by a hydrophobic region) typical of proteins that are secreted by the twin-arginine translocation (Tat) pathway (1).

In case of *cuoC*, two different start codons could be chosen. One of them was a CTG codon with a putative ribosome-binding site (GAGGG) 4 bp upstream; the other was an ATG codon with no appropriate candidate ribosome-binding site. The distance between both codons was only 12 bp, with CTG being located upstream of ATG. However, the predicted secretion system used to export the protein would be different

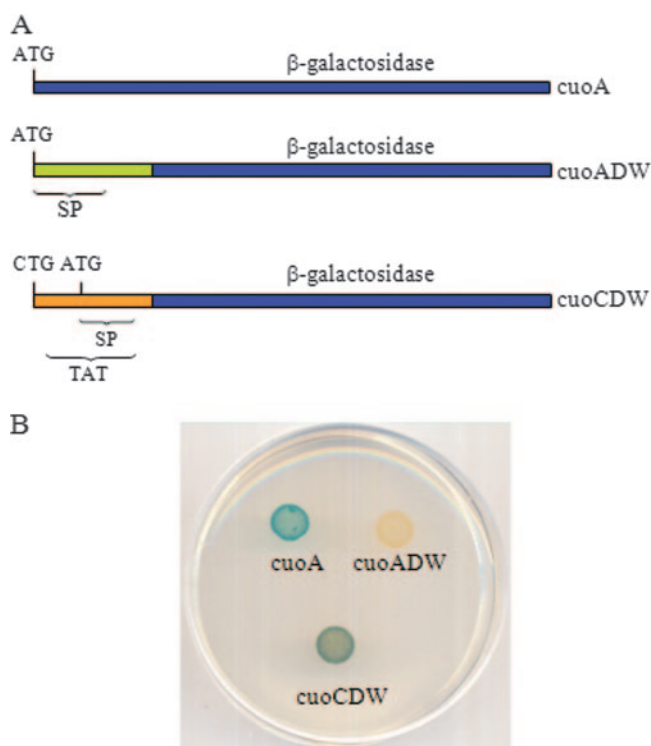


FIG. 4. Determination of the start codon of *cuoC* and the secretion systems used by CuoC and CuoA. (A) Relevant features of the three fusions constructed between *cuoA* or *cuoC* and *lacZ*. The construct *cuoA* is harbored in strain JM51AZY, *cuoADW* in JM51ADWZY, and *cuoCDW* in JM51CDWZY (see Table 1 for details). (B) Analysis of  $\beta$ -galactosidase activity in the bacteria harboring the three fusions mentioned above. These bacteria were incubated for 24 h on CTT agar plates containing 600  $\mu\text{M}$  copper sulfate and 100  $\mu\text{g/ml}$  X-Gal.

depending on the start codon used: the protein would be secreted by the Tat system if CTG were the start codon, but it would be secreted by the Sec system in the case of its being ATG. In order to test the secretion system used by CuoC, a translational fusion between *cuoC* and *lacZ* from *E. coli* was constructed (strain JM51CDWZY). The fusion was performed at a codon located after both signal secretion signatures (Fig. 4A), so the chimeric protein would be secreted to the periplasmic space. It is known that the Tat system translocates proteins that are completely folded, while the Sec system secretes unfolded protein (2). It is also known that  $\beta$ -galactosidase has to be properly folded in a tetrameric form in order to be active (13). Therefore, the chimeric protein would be active if secreted by the Tat system, but it would be inactive if secreted by the Sec system. When this experiment was performed, it was observed that the chimeric protein was active (see Fig. 4B). As controls, we constructed two translational fusions between *cuoA* and *lacZ*, one at the predicted start codon (strain JM51AZY) and the other at a codon after the signal peptide sequence (strain JM51ADWZY), as depicted in Fig. 4A. In this case, the fusion at the start codon was active, while the chimeric protein was inactive (Fig. 4B), indicating that CuoA is indeed secreted by the Sec system. We therefore concluded that CuoC is secreted by the Tat system and that CTG func-

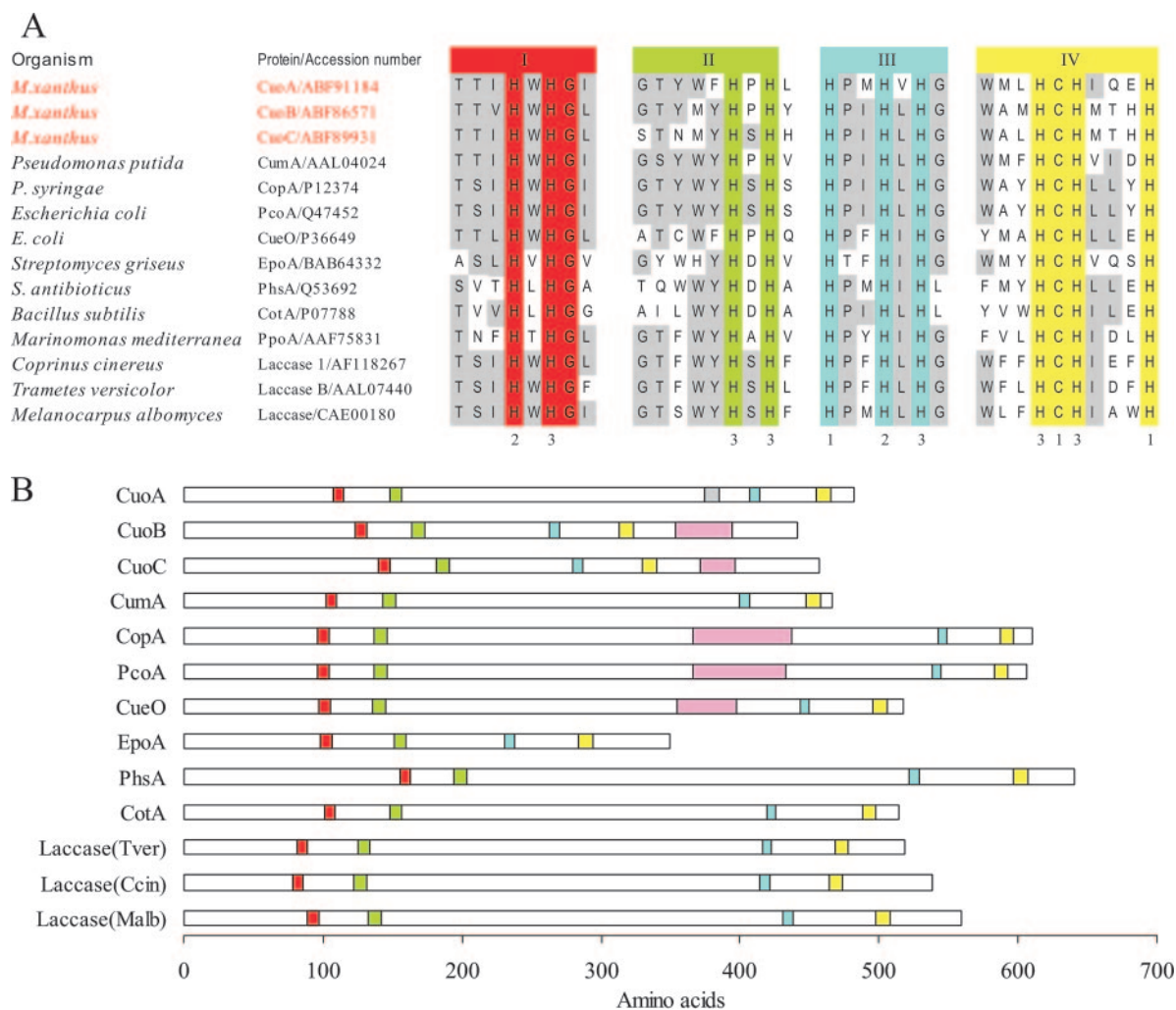


FIG. 5. Domain organization of *M. xanthus* MCOs. (A) Alignments of the four copper domains of CuoA, CuoB, and CuoC of *M. xanthus* with those of other bacterial and fungal MCOs. Histidine and cysteine residues involved in the coordination of copper atoms are numbered according to the copper type. Invariant residues in all the proteins are shown on a red, green, blue, or yellow background for motif I, II, III, or IV, respectively, whereas conserved residues in at least six proteins are shaded in gray. (B) Schematic representation of the motifs in *M. xanthus*, fungal, and bacterial MCOs. The four copper domains are represented with the same color as in panel A. The gray rectangle in CuoA represents a histidine-rich motif. Methionine-rich motifs in several MCOs are represented by pink rectangles.

tions as the start codon for *cuoC*, with the twin-arginine motif being RRSML. This gene encodes a protein with 461 residues.

The three MCO genes are located nearby in the chromosome in a 26.5-kb region that codes for 20 proteins, including at least 9 with conserved domains known to be involved in copper handling and trafficking. Figure 5A shows the amino acid sequence alignments of the four predicted copper-binding domains from *M. xanthus* MCOs, characterized bacterial MCOs, and crystallized fungal laccases. The three *M. xanthus* proteins contain perfect matches with the sequence signature motifs for MCOs. However, CuoA is quite different from CuoB and CuoC, which are at the same time very similar to one another. Thus, the distance between motifs II and III is shorter in CuoB and CuoC than in CuoA (Fig. 5B). CuoA exhibits only 24.45 and 22.91% identity with CuoB and CuoC, respectively, while this parameter rises to 56.18% when CuoB and CuoC are compared. Another difference is that only CuoA

exhibits a histidine-rich region between domains II and III (Fig. 5B), a peculiarity that has not been observed in the other MCOs. CuoB and CuoC, on the contrary, exhibit methionine-rich motifs in their C-terminal domains.

#### Expression profiles of *cuoA*, *cuoB*, and *cuoC* during growth.

To get insights into the expression of the three MCO genes, three different *M. xanthus* strains harboring a fusion between *cuoA*, *cuoB*, or *cuoC* and *lacZ* from *E. coli* were constructed.

In the absence of copper, the expression of *cuoA* and *cuoB* remained undetectable (Fig. 6A and B). On the contrary, basal expression levels for *cuoC* were around 200 units (Fig. 6C).

The three promoters were highly dependent on the addition of external copper, although the amount of metal and the incubation times required for maximum induction were clearly different for each gene (Fig. 6). In the case of *cuoB*, the maximal expression level was higher with 300  $\mu$ M copper than with 600  $\mu$ M, indicating that the *cuoB* promoter responds very

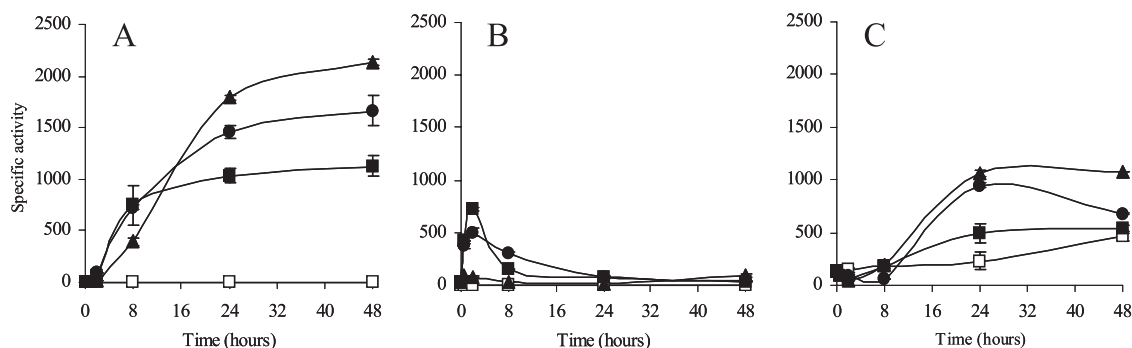


FIG. 6. Copper up-regulation of *cuoA* (A), *cuoB* (B), or *cuoC* (C) promoter during growth. Strains JM51AZY, JM51BZY, and JM51CDWZY (see Table 1) were incubated on CTT agar plates containing 0 (□), 300 (■), 600 (●), or 800  $\mu$ M (▲) copper sulfate.  $\beta$ -Galactosidase specific activity in cell extracts was determined as described in Materials and Methods. Error bars indicate standard deviations.

efficiently to lower copper concentrations (Fig. 6B). The *cuoA* promoter was induced in a stepwise fashion as the external Cu(II) ions were increased, reaching the maximum levels at subinhibitory copper concentrations (Fig. 6A). The promoter of *cuoC*, on the contrary, was induced only at high copper concentrations (Fig. 6C). As a summary, the *cuoB* and *cuoC* promoters seemed to respond differentially to low and high copper concentrations, respectively, while *cuoA* responded to a broad range of copper concentrations, exhibiting a linear dependency on the external copper.

The maximum expression time for each gene was also clearly different. The early induction of *cuoB*, reaching a peak 2 h after the metal addition, suggests that this MCO forms part of the primary copper response (Fig. 6B). With longer incubation times, expression decreased nearly to prestress levels after 24 h. In the case of *cuoA* and *cuoC*, the expression increased with time, reaching a plateau between 24 and 48 h of incubation with copper (Fig. 6A and C).

**Expression of *cuoA*, *cuoB*, and *cuoC* during development.** To analyze the MCO promoter responses to copper during development, a 10-fold-lower concentration of the metal had to be used in order to prevent cells from dying (Fig. 2A). Very similar to the case with growing cells, *cuoA* and *cuoB* were not expressed in the absence of copper (Fig. 7A and B). However, *cuoC* expression profiles changed under developing conditions, and this promoter exhibited a copper-independent induction by starvation (Fig. 7C).

The copper-responsive expression profile for *cuoA* was similar to that shown during growth, although with only 1/10 of the metal in the medium. That means that *cuoA* exhibited a linear induction dependent on the external copper concentration (Fig. 7A). On the contrary, several differences could be observed in the expression of *cuoB* during development. First, the induction was not so immediate, and the time of maximal induction changed from 2 h during growth to 8 h (Fig. 7B). Second, all the concentrations tested exhibited similar expression levels. As in the case of *cuoA*, a 10-fold-lower amount of copper was required to achieve maximum expression during development. In contrast, the *cuoC* promoter did not respond to the addition of metal during development, even with copper concentrations as high as 60  $\mu$ M (Fig. 7C).

**Role of MCOs in copper resistance.** To analyze whether the three MCOs are involved in copper tolerance, three in-frame deletion mutants were constructed (Table 1). The three mutants and the WT strain were incubated in liquid CTT medium containing several copper concentrations. As shown in Fig. 1A, the copper tolerances for  $\Delta$ *cuoA* and  $\Delta$ *cuoC* mutants were very similar to that of the WT strain. On the contrary, the  $\Delta$ *cuoB* mutant was markedly more sensitive to copper than the other three strains, as no growth was observed with copper concentrations of 500  $\mu$ M.

Although the  $\Delta$ *cuoA* mutant and the WT strain exhibited similar growth rates with 700  $\mu$ M copper, optical, transmission, and scanning microscopy analyses revealed that the mutant

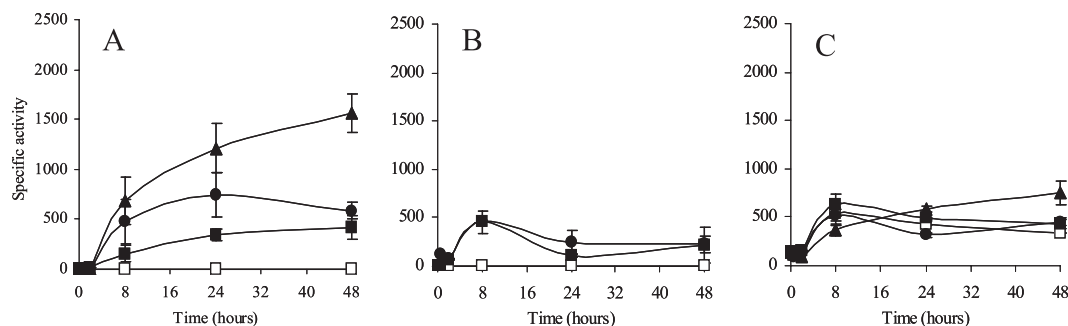


FIG. 7. Copper up-regulation of *cuoA* (panel A), *cuoB* (panel B), or *cuoC* (panel C) promoter during development. Strains JM51AZY, JM51BZY, and JM51CDWZY (see Table 1) were incubated on CF agar plates containing 0 (□), 20 (■), 40 (●) or 60  $\mu$ M (▲) copper sulfate.  $\beta$ -Galactosidase specific activity in cell extracts was determined as described in Materials and Methods. Error bars indicate standard deviations.



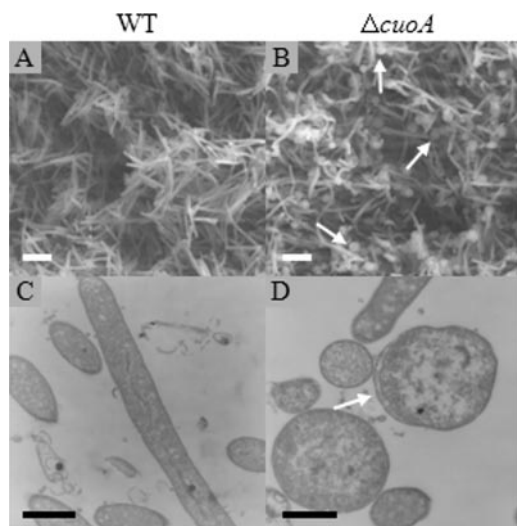


FIG. 8. Morphology and ultrastructure of vegetative cells of the WT (A and C) or  $\Delta cuoA$  (B and D) strain grown on CTT medium containing 700  $\mu\text{M}$  copper sulfate. Pictures were taken with a SEM (A and B) or TEM (C and D) after 72 h of incubation. Bars represent 3  $\mu\text{m}$  in panels A and B or 0.5  $\mu\text{m}$  in panels C and D. Arrows point to spherical forms (B) or a region where the distance between the outer and inner membranes is unusual (D).

cells exhibited altered morphologies, since many of them (approximately one-third) became spherical instead of remaining typical long rods (Fig. 8). The number of spherical forms increased with higher copper concentrations for the mutant. Some of these forms could also be observed for the WT strain, but only with 900  $\mu\text{M}$  copper. We were also able to detect spherical forms for the  $\Delta cuoC$  mutant grown with 700  $\mu\text{M}$  copper but at a much lower ratio than for the  $\Delta cuoA$  strain.

We obtained two contradictory observations with the  $\Delta cuoA$  and  $\Delta cuoC$  strains: mutant cells exhibited alterations in morphology, but they could tolerate the same metal concentration as the WT strain or even higher. Although other alternatives cannot be ruled out, one possible explanation for these paradoxical observations could be that the lack of CuoA or CuoC might prevent a proper detoxification of the periplasmic space, provoking the disruption of the peptidoglycan. Simultaneously, this lack of detoxification might induce other mechanisms involved in copper resistance, enough to maintain or even increase viability of cells but not to detoxify the periplasmic space. As a result, the mutant cells would survive in the presence of high copper concentrations, like the WT cells, but not as long rods; rather, as spherical forms. If this premise is correct, mutant cells that have been grown in the presence of copper to induce all the mechanisms involved in resistance should be more sensitive to the metal than the WT cells. To test this hypothesis,  $\Delta cuoA$  and  $\Delta cuoC$  mutants and the WT strain were grown in the presence of 600  $\mu\text{M}$  copper sulfate (to induce all of the copper resistance mechanisms) and then diluted into fresh medium containing higher copper concentrations. As shown in Fig. 1B, the  $\Delta cuoA$  mutant was more sensitive to copper than the WT strain under these conditions, which is consistent with our initial hypothesis. On the other hand, we could not observe a clear decrease in sensitivity in the case of the  $\Delta cuoC$  mutant.

**Role of MCOs in development.** When no copper was added to the starvation CF medium, all mutants exhibited a delay in aggregation compared to the WT strain (data not shown). However, after 72 h of incubation, the mutant fruiting bodies were very similar to those of the WT strain. Only the  $\Delta cuoC$  fruiting bodies exhibited some abnormalities in morphology (Fig. 2A). The number and ability to germinate of the myxospores obtained in the absence of metal were similar in the  $\Delta cuoA$  and  $\Delta cuoB$  mutants compared to the WT strain (data for WT and  $\Delta cuoA$  mutant are shown in Fig. 3A and B). On the contrary, the yield of myxospores of the  $\Delta cuoC$  mutant decreased to one-third of that of the WT strain (Fig. 3A). In addition, the ability of these mutant spores to germinate was even more reduced. As shown in Fig. 3B, only 0.39% of the  $\Delta cuoC$  myxospores were able to germinate, versus 11% obtained in the case of the WT strain (Fig. 3B).

In the presence of copper, the  $\Delta cuoB$  mutant was much more sensitive than the WT strain, as was observed during growth. As shown in Fig. 2A, this mutant could make fruiting bodies only with copper concentrations up to 20  $\mu\text{M}$ . With greater concentrations, cells died. The capacity of spore formation and germination remained unaltered in this mutant compared to the WT in the presence of 20  $\mu\text{M}$  of copper (data not shown). The  $\Delta cuoA$  and  $\Delta cuoC$  mutants not only aggregated at higher copper concentrations than the WT strain but also were able to reshape and originate myxospores. The yield of myxospores in the  $\Delta cuoA$  mutant and the WT strain was decreasing at the same rate as the copper concentration was increasing (Fig. 3A). However, the  $\Delta cuoA$  mutant exhibited a greater germination efficiency, which is consistent with the fact that these mutant cells are more resistant to copper. As shown in Fig. 3B, only 0.3% of the WT myxospores obtained with 60  $\mu\text{M}$  copper were able to germinate, versus 4.4% in the case of the  $\Delta cuoA$  mutant. The  $\Delta cuoC$  strain yielded even more myxospores with 60  $\mu\text{M}$  copper than in the absence of metal (Fig. 3A). But this mutant was clearly affected in germination, and only 0.032% of the spores obtained with that copper concentration were able to originate colonies in rich medium (Fig. 3B). Moreover, the  $\Delta cuoA$  and  $\Delta cuoC$  mutants yielded myxospores with the ability to germinate with 80  $\mu\text{M}$  copper (Fig. 3).

When cells inside the aggregates of the three mutants were compared to those of the WT strain under a TEM, it was observed that  $\Delta cuoB$ ,  $\Delta cuoC$ , and WT myxospores were identical at all the copper concentrations that could be tested (Fig. 9A, C, D, and F). The  $\Delta cuoC$  cells maintained an unaltered spore coat even at 80  $\mu\text{M}$  copper (Fig. 9I). In the case of the  $\Delta cuoA$  mutant, the external coat gradually became fainter as the copper concentration increased, completely disappearing with 80  $\mu\text{M}$  copper (compare panels B, E, and H in Fig. 9). These myxospore-like cells obtained with 80  $\mu\text{M}$  copper were different from the spherical forms observed during vegetative growth for this mutant. First, myxospore-like cells were observed only in the aggregates and not in the peripheral rod population surrounding the fruiting bodies. Second, the same type of inclusion bodies as those observed in the typical myxospores appeared inside these spherical cells (Fig. 9). Third, some of these cells were able to germinate.

In concordance with the phenotype observed during growth, we assayed development of  $\Delta cuoA$  and  $\Delta cuoC$  mutants when



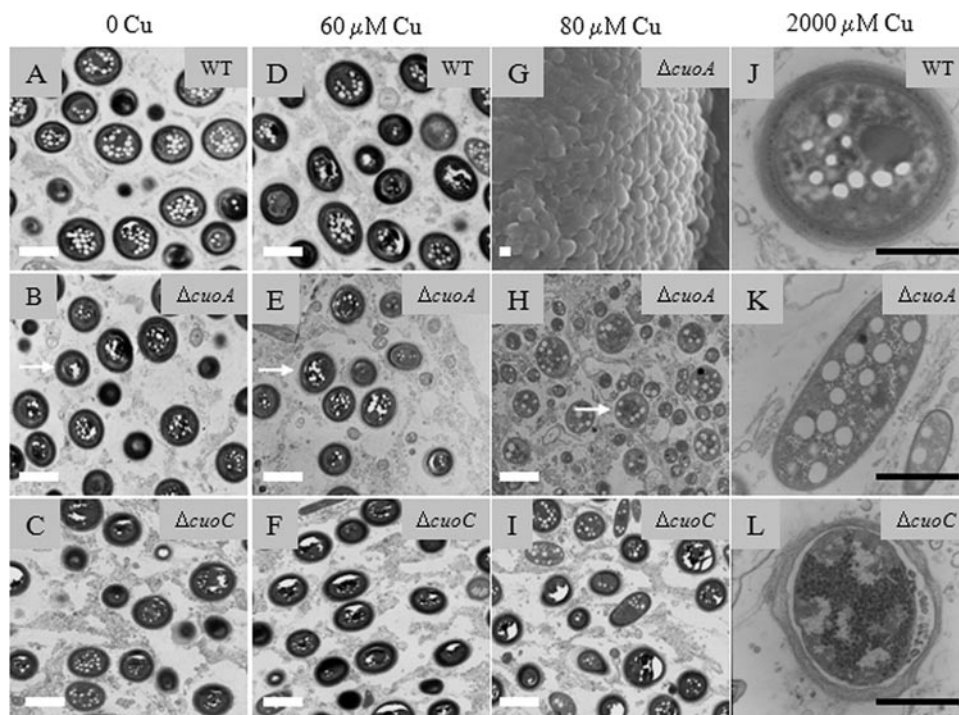


FIG. 9. Effect of copper on the spore coat. Fruiting bodies from WT,  $\Delta cuoA$ , and  $\Delta cuoC$  strains were harvested from CF agar containing the indicated copper sulfate concentrations and treated as described in Materials and Methods. All the pictures were taken with a TEM, with the only exception being panel G. Picture G, taken with a SEM, shows a fruiting body filled with myxospores formed by the  $\Delta cuoA$  mutant at 80  $\mu\text{M}$  of copper sulfate. Myxospores formed by copper-preadapted cells on CF containing 2,000  $\mu\text{M}$  copper sulfate are shown in pictures J (WT strain) and K ( $\Delta cuoA$  mutant). In the case of the  $\Delta cuoC$  mutant (L), a copper concentration of 1,500  $\mu\text{M}$  was used, because with greater concentrations this mutant does not make fruiting bodies. White bars, 1  $\mu\text{m}$ ; black bars, 0.5  $\mu\text{m}$ . Arrows in panels B, E, and H point to the outer coat of the myxospores.

cells were previously grown with 600  $\mu\text{M}$  copper. In these experiments, the  $\Delta cuoA$  mutant also turned out to be more sensitive to copper than the WT strain. Under these circumstances, the  $\Delta cuoA$  mutant formed fruiting bodies only up to 2,000  $\mu\text{M}$ , versus the WT strain, which was able to develop with a 2,500  $\mu\text{M}$  concentration (Fig. 2B). Mutant cells died at higher copper concentrations. In addition, the spore ultrastructure was absolutely different in this mutant. As shown in Fig. 9K, the coat of cells inside the aggregates disappeared. Furthermore, the morphology of the mutant cells was also affected, and more than 90% failed to completely reshape, remaining short rods. Contrary to the case with the mutant, most of the WT spores exhibited an unaltered cell envelope (Fig. 9J).

In the case of the  $\Delta cuoC$  mutant, preincubated cells exhibited the same viability as the WT strain (swarming was observed, and cells grew when transferred to rich medium). However, they were unable to aggregate and sporulate with copper concentrations higher than 1,500  $\mu\text{M}$  (Fig. 2B). Spores obtained with 1,500  $\mu\text{M}$  copper were covered with a deteriorated spore coat (Fig. 9L).

**Cuprous oxidase activity in *M. xanthus* cell extracts.** MCOs have been reported to oxidize a wide variety of aromatic compounds (phenolics and nonphenolics) and also metals, such as cuprous ions. To investigate how *M. xanthus* MCOs exert their functions, we searched for several enzymatic activities in WT cell extracts obtained from cells grown in the absence or pres-

ence of copper. With use of cell crude extracts, no oxidation activity was detected towards the oxidation of any the aromatic compounds tested, either in cells grown in the absence of copper or in those incubated with this metal. Instead, we were able to measure cuprous oxidase activity in crude extracts of WT and mutant strains from cells harvested after 24 h of growth in the absence of copper. Furthermore, significant changes were found in cell extracts grown in the presence of increasing concentrations of copper (Fig. 10). WT cells grown in the presence of 300 or 600  $\mu\text{M}$  copper exhibited a 2.7- or 3.4-fold increase in the enzymatic rate, respectively, compared with cells grown in the absence of copper. In addition, cuprous activity in cell extracts from the  $\Delta cuoA$  mutant at all of the copper concentrations tested and the  $\Delta cuoC$  mutant at 600  $\mu\text{M}$  was clearly reduced. This reduction was not observed with the  $\Delta cuoB$  strain, although it should be noted that the expression of the *cuoB* promoter in the WT strain at this growth stage was negligible (Fig. 6). These results are in accordance with the observed copper up-regulation response of the MCO genes.

## DISCUSSION

The copper response has been deeply studied so far with two bacteria, *E. coli* and *Enterococcus hirae* (33, 37, 39), but the elucidation of the copper systems in *M. xanthus* will provide a missing piece in the complicated puzzle of bacterial adaptation to this metal under natural environmental conditions: how do

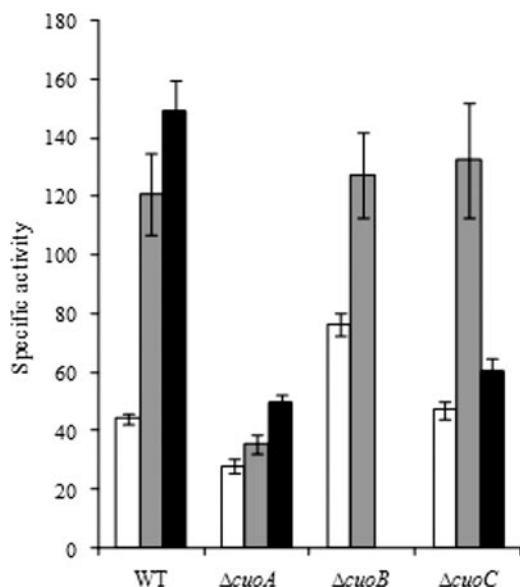


FIG. 10. Cuprous oxidase activity in crude extracts of WT and mutant strains harvested after 24 h of growth in absence (white bars) or presence of 300 (gray bars) or 600  $\mu$ M (black bars) copper sulfate.

cells handle different copper concentrations during a complete life cycle?

In this report we have first studied the effect of copper on developing cells, and we have found that they are approximately 15-fold more sensitive to this metal than growing cells. It should be noted that developing cells have to undergo many changes in the cell envelope in order to reshape and become myxospores. These alterations could make developing cells extremely sensitive to copper stress. Additionally, these cells are physiologically different. As they are starving, their energy levels are considerably lower than those of vegetative cells. It has been reported for *E. coli* that anaerobic cells are also more sensitive to copper than those that are grown in the presence of oxygen (30). These authors have proved that growth in the absence of oxygen clearly alters the copper physiology and that two different systems involved in copper tolerance are differently regulated during aerobic and anaerobic growth. The data obtained for *E. coli* and those reported in the present study on *M. xanthus* during development and growth indicate that the physiological stage of cells is an important factor in copper tolerance.

However, *M. xanthus* copper-preadapted cells are as resistant to this metal during development as during growth. Elucidation of this huge increase in resistance during development probably will reveal a very complex and intricate process, where many different proteins will be involved. Our data indicate that once the copper adaptation mechanisms are turned on, cells are able to tolerate higher copper concentrations. In fact, we have demonstrated that the induction of *cuoA* and *cuoC* (and, therefore, the resistance conferred by CuoA and CuoC) persists during long periods of time.

Due to the large variety of functions where MCOs seem to be involved, we have investigated the role that the three MCOs from *M. xanthus* play during growth and development in an attempt to find the reasons for such a high gene redundancy in

the chromosome. From the sequence data analyses and translocation systems used by the three MCOs, we can distinguish two groups: CuoA and CuoB/CuoC. CuoB and CuoC possess the methionine-rich region proximal to the copper centers found in all of the MCOs with a function in copper resistance. This domain has been suggested to function as a regulator that binds copper to activate the enzymes or as a mediator in protein-protein interaction (34). In addition, they are secreted by the Tat pathway, which transports fully folded proteins with their cofactors bound before translocation into the periplasm (2). These two features indicate that these two MCOs will eliminate copper from the cytoplasm during translocation. On the contrary, CuoA lacks this methionine-rich region, and it is translocated by the Sec system, what means that this protein has to incorporate copper as a cofactor into the periplasm. These differences reinforce our interest in the compared study of regulation and physiological role of these proteins.

One of the reasons for MCO redundancy must be the differences in the expression profiles exhibited by each gene during growth and development. A first difference observed is that *cuoC* is the only MCO expressed during growth and development in the absence of copper. Although the three promoters are induced by this metal during growth, the timing and copper levels required for maximum expression differ for each gene. These data, along with the phenotypic analyses of deletion mutants, indicate that each gene is specialized in a different function. CuoB seems to be involved in the primary adaptive response to copper; hence, its promoter responds rapidly after copper addition, and its expression decreases as the expression of the other two genes increases. As a result,  $\Delta$ *cuoB* mutant cells are extremely sensitive to this metal. On the contrary, CuoA and CuoC seem to be responsible for the maintenance of the response. They are induced more slowly than CuoB, and their expression levels reach a plateau. These results clearly demonstrate that along with differential induction with increased copper concentrations, differences in timing of expression to adjust the cells to the Cu-regulated adaptive response justify the redundancy of the three MCOs in *M. xanthus*.

One interesting observation is that the induction of *cuoA* and *cuoB* during development requires a 10-fold-lower amount of copper than during growth to reach similar levels of expression. This difference in promoter sensitivity has not been observed either with *cuoC* or with genes involved in carotenoid biosynthesis (unpublished results). On the contrary, *cuoC* is induced by starvation. An explanation for these differences could be that the three genes are regulated by two different mechanisms, one functioning during development and the other during growth. A multiple regulation has been previously described for several proteins involved in the copper response. Hence, the Cu(I)-translocating P-type ATPase CopA, which is the central component in copper homeostasis in *E. coli*, is regulated by the metal-responsive regulator CueR, but its expression is also influenced by the cell envelope stress response regulator CpxR (31). In addition, the oxygen sensor FNR also induces *copA* expression under microaerobic conditions (32). Only the identification of the factors involved in the regulation of the three *M. xanthus* MCOs will answer these intriguing questions.

Phenotypic analysis of the  $\Delta$ *cuoA* deletion mutant revealed that the morphology of one-third of the mutant cells dramati-

ically changes during growth with copper, and the long rods become spherical forms (Fig. 8). This change in shape suggests a loss of peptidoglycan, a phenotype that has not been reported for any other bacteria where the copper response has been studied, such as *E. coli*. The different behavior of the *M. xanthus*  $\Delta$ *cuoA* mutant cells can be attributed to the peculiarities of peptidoglycan in this myxobacterium. This peptidoglycan consists of patches that are connected by bridges that are sensitive to treatments such as sodium dodecyl sulfate and proteases (8). These bridges must also be sensitive to the oxidative stress originated by copper, causing the loss of the morphology of the mutant cells in the presence of this metal. Since this phenotype is not observed so drastically with the *cuoB* and *cuoC* mutants, probably *CuoA* is the main MCO responsible for periplasmic detoxification. This hypothesis is further reinforced by the lack of a spore coat in the  $\Delta$ *cuoA* myxospores obtained with 80 and 2,000  $\mu$ M copper. The spore coat has to be secreted from the cytoplasm to the exterior surface, and during this traffic, materials have to go across the periplasm. They are there subjected to copper deleterious side reactions in the *cuoA* mutant, preventing proper assembly and originating myxospores with no coat.

The MCO gene *cotA* from *B. subtilis* encodes a structural component of the outer coat but is not copper inducible (24). *CuoC* could be a good candidate to exert a function similar to that of *CotA* in the myxospore coat because of the regulation of the gene upon starvation in the absence of extracellular copper. However, several evidences point in another direction. Thus, this gene is expressed at quite high basal levels and is induced by copper during growth. Therefore, if *CuoC* were located in the spore coat as is *CotA*, this protein should exert another function during growth in the presence of high copper concentrations. Nevertheless, *CuoC* seems to have an important role during development, because severe defects are observed in the mutant strain, with or without copper. We believe that all of these defects most likely can be attributed to a lack of detoxification in the mutant cells.

The role of *CueO* from *E. coli* in copper homeostasis is difficult to discern due to the fact that this protein is able to oxidize a broad range of substrates, such as cuprous compounds, catechols, and other aromatic compounds or iron-chelating siderophores (15, 35). From this perspective, it could also be discussed that the coexistence and expression of three MCOs in *M. xanthus* are due to different enzymatic activities exerted by these proteins. This question will be clarified only by biochemical characterization of the three purified enzymes, an investigation that is currently under way in our laboratories. However, the results obtained from oxidase assays with *M. xanthus* WT and mutant cell extracts have revealed that most likely they will exert their functions through cuprous oxidase activity. The *cuoA* promoter exhibits not only the highest levels of expression but also the strongest dependency on the copper concentration in the medium. Accordingly, the deletion of this gene in the  $\Delta$ *cuoA* mutant results in the lowest enzymatic activities measured together with the lowest activity dependency on external copper addition. Therefore, the kinetic analysis supports an essential role for this enzyme in the full copper tolerance of *M. xanthus* during vegetative growth, presumably through Cu(I) detoxification in the periplasmic space (Fig. 1B). Furthermore, the levels of enzymatic activities in the  $\Delta$ *cuoB*

and  $\Delta$ *cuoC* mutants confirm the weak involvement of *CuoB* in copper responses at late stages of growth and a major contribution of *CuoC* during vegetative growth at higher copper concentrations.

As a summary, we conclude that the three paralogous genes that encode MCOs in *M. xanthus* are differentially regulated; that *CuoA* is mainly involved in periplasm detoxification and is necessary for full copper tolerance during growth and development; that *CuoB* is essential in the primary adaptive response to copper oxidative stress; and that *CuoC* plays an important role in development, especially in the sporulation process. These different functions might explain why *M. xanthus* requires three MCO paralogs.

#### ACKNOWLEDGMENTS

This work was supported by Ministerio de Educación y Ciencia (grants BMC2003-02038 and BFU2006-00972/BMC, 70% funded by FEDER, and the integrated action HP1005-0034). M.C.S.-S. and N.G.-S. are predoctoral fellows from Plan Propio, Universidad de Granada. A.M.-M. was granted a postdoctoral fellowship from Universidad de Granada.

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