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Copper accumulation in *Xanthomonas campestris* pv. vesicatoria

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ABSTRACT

A strain of *Xanthomonas campestris* pv. vesicatoria showing resistance to 1.2 mM cupric sulfate was analyzed by atomic absorption spectroscopy and ESI (electron spectrophotometry imaging). Accumulation of copper was detected in the periphery of the cell membrane region, suggesting that the mechanism of copper resistance is similar to that previously described for *Pseudomonas* species. The ESI technique was used to detect copper in the membrane region. Copper-resistance in *X. campestris* pv. vesicatoria 484 is inducible and occurs by accumulation of the metal and not by efflux mechanism as has been suggested. The growth curve also showed that this system is inducible.

INTRODUCTION

Bacterial leaf spot of tomato and pepper caused by *Xanthomonas campestris* pv. vesicatoria is a problem, especially in tropical regions with high temperature and humidity. Control of this disease is provided mainly by the employment of bactericides based upon copper and antibiotics such as streptomycin. However both compounds have become less effective due to the development of resistance by *Xanthomonas* strains (Stall and Thayer, 1962; Marco and Stall, 1983). Resistance to copper was first detected in Florida and later was also registered in Oklahoma and California (Bender *et al.*, 1990; Cooksey *et al.*, 1990). The copper-resistance gene has also been described in *Pseudomonas* (Bender and Cooksey, 1986, 1987) and was genetically analyzed and sequenced (Mellano and Cooksey, 1988a,b). Recently, Voloudakis *et al.* (1993) have shown that the copper-resistance genes of different isolates of *X. campestris* pv. vesicatoria are closely related to each other and all showed similarity to the *cop*A gene of *Pseudomonas.* Preliminary studies on Brazilian isolates of *X. campestris* pv. vesicatoria have shown high levels of copper resistance (Aguiar *et al.*, 1994), however DNA similarity analyses were not performed.

Copper-resistance mechanisms in bacteria were recently reviewed (Brown *et al.*, 1992; Silver and Walderhaug, 1992; Cooksey, 1993) and although different mechanisms have been suggested, the evolutionary paths seem limited (Cooksey, 1993). Basically, it has been suggested, that an energy-dependent mechanism of copper efflux occurs in *Escherichia coli* (Rouch *et al.*, 1985; Brown *et al.*, 1992) and that an accumulation of copper in the periplasm and outer membrane occurs in *P. syringae* (Bender and Cooksey, 1987; Cha and Cooksey, 1991) and other *Pseudomonas* species (Cooksey and Azad, 1992). Voloudakis *et al.* (1993) have suggested an efflux mechanism in *X. campestris* pv. vesicatoria, as in *E. coli*, to explain the copper resistance, based upon the lack of blue colored colonies when the strains are grown in high concentrations of copper.

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MATERIAL AND METHODS

Bacterial strains and MIC (minimum inhibitory concentration) determination

X. campestris pv. vesicatoria 484 and 457 were isolated from tomato plants by J. Rodrigues Neto, Biological Institute, Campinas, Brazil. Bacteria were grown in NA medium (beef extract 0.3%, peptone 0.5%) with or without agar (1.5%) at 28°C. MICs were determined according to Cooksey *et al.* (1990).

Induction of copper resistance

The copper-resistant (484) and -sensitive (457) strains were grown overnight in NA medium with and without 0.2 mM cupric sulfate. One ml of these cultures was used as an inoculum for 20 ml of medium supplemented with 1.2 mM cupric sulfate. Samples were taken at intervals of 30 min during 2.5 h and thereafter each 2 h. Growth was measured as absorbance in a spectrophotometer (A_{600}).

Copper accumulation measurements

The copper-resistant strain (484) was grown overnight in 500 ml of NA medium containing 0.8 mM cupric sulfate. The cellular suspension was adjusted to an optical density of 0.6 (A_{600}). Acid digestion was performed according to Ganje and Page (1974) and Nkong and Ballance (1982). The suspension was centrifuged (7,000 rpm/15 min) and the pellet resuspended in 10 ml sterile milli-Q water. Aliquots of 1.3 ml were transferred to glass tubes and 2.0 ml of H_2SO_4 (86%) was added. The reaction was maintained in a digestion block at 120°C until clarification of the sample (around 24 h). After cooling the sample, 100 µl of H_2O_2 (30%) was added to complete oxidation and the tube was maintained in the digestion block for 30 min. The final volume was adjusted to 100 ml with sterile milli-Q water. All the experiments were carried out in duplicate. The control was processed in the same way except for omission of the cupric sulfate in the growth medium. To measure the copper accumulation, a standard curve using concentrations from 1 to 11 g/ml of cupric sulfate was constructed. The measurement of copper was carried out in an FMD3 Zeiss atomic absortion spectrophotometer adjusted to 324.8 nm and calibrated.

Electron spectroscopy imaging (ESI)

The bacteria were grown as mentioned above using 50 ml of medium. The cells were pelleted, resuspended in 0.1 M phosphate buffer, pH 7.4 (Buffer I), pelleted again and resuspended in Buffer I + 2% glutaraldehyde and pre-fixed during 1 h at 4°C. The cells were pelleted and resuspended in Buffer I (2 x). This procedure was repeated twice. In the last centrifugation, the pellet was fixed in Buffer I + 1% osmium tetroxide for 10 min at 4°C. The cells were pelleted, washed in sterile distilled water and finally set in 1.5% agar-water blocks. The blocks were dehydrated in acetone: 30, 50, 70, 90 and 100% (2 x), for 10 min. The infiltration was in acetone: resin (1:1) for 24 h and resin at 70°C for 5 days. The material was set in Epon-Araldite during 3 days at 70°C. Sections of 60 nm were taken and analyzed at low (910 eV) and high (931 eV) energy using a Zeiss CEM 902 transmission electron microscope coupled to an electron spectroscopy imaging system. The image was processed by IBAS computer software.

RESULTS AND DISCUSSION

In a preliminary experiment 12 strains of *X. campestris* pv. vesicatoria, isolated in Brazil, were tested for copper resistance in different concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mM) of cupric sulfate. Fifty percent of these strains showed high or moderate levels of copper resistance. The 484 strain showed a more intense ochre color when grown on copper-supplemented medium, which suggested accumulation of the metal. This color change was not described in *Xanthomonas* copper-resistant strains, however it was observed for *P. syringae* strains and several other copper-resistant and copper-accumulating species of *Pseudomonas* (Cooksey and Azad, 1992; Voloudakis *et al.*, 1993). Therefore 484 strain was used for further studies. The sensitive strain 457 grew on medium containing up to 0.2 mM cupric sulfate.

The copper-resistant strain (484) exhibited different growth curves, depending upon the growth conditions of the inoculum (Figure 1). If the previous growth occurred in a medium containing a low concentration of copper (0.2 mM), the growth curve was identical to that of strain 484 grown in the absence of copper (curves A and C). However, if it has not been previously exposed to copper, there was a lag time of approximately 12 h before normal growth started (curve B). This lag time indicated that an induction period was necessary to permit the expression of

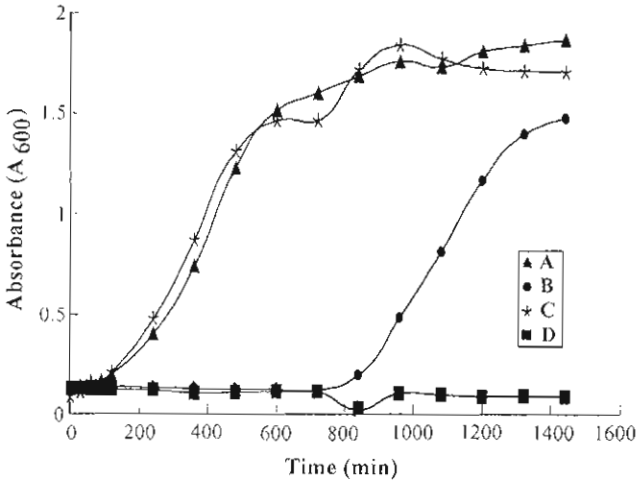

Figure 1 - Growth curve of *Xanthomonas campestris* pv. vesicatoria strain 484 (resistant) and 457 (sensitive) in the presence and absence of copper. A: Strain 484 pre-cultured in the presence of 0.2 mM cupric sulfate and grown in the presence of 1.2 mM cupric sulfate. B: Strain 484 grown as A, but pre-cultured in the absence of copper. C: Strain 484 without copper. D: Strain 457 in 1.2 mM cupric sulfate.

resistance genes and it was completely eliminated if the strain had been previously cultured in the presence of copper. The sensitive strain (457) showed no growth at all (curve D), as expected. The inductive system for copper-resistance genes has been previously described in *E. coli* (Rouch *et al.*, 1985) and *P. syringae* (Mills *et al.*, 1993). However, the lag time was variable, depending on the bacterial strain and the type of heavy metal resistance gene under analysis. Although the system is inducible in *X. campestris* pv. vesicatoria we were unable to detect proteins specifically induced by copper using polyacrylamide gel electrophoresis and silver staining (data not shown). Possibly the proteins are synthesized at a low level and detectable only by immunoblotting. Cooksey *et al.* (1990) detected two or more proteins, as faint bands, in Western blots, indicating a low level expression of these proteins.

The electron spectroscopy imaging (ESI) analysis was carried out with the resistant strain grown in the presence and absence of copper. The cells grown in the presence of copper presented uniform color when low electron transmission energy was used (Figure 2, top) whereas a white color could be seen around the cells when copper-specific energy was applied (Figure 2, bottom). This white color in the cellular peripheric region indicates accumulation of the metal. Also the measurements of copper by absorption spectrophotometry indicated accumulation of 10.83 µg/ml of the metal, whereas none was found in the control. Copper accumulation in the membranes was described for *Pseudomonas* species (Cooksey and Azad, 1992). The *cop*

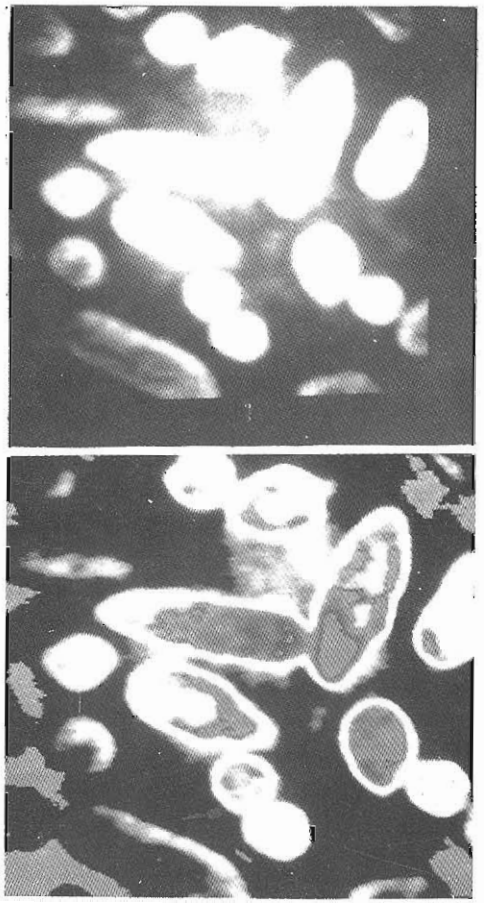

Figure 2 - Selective imaging with ESI (electron spectroscopy imaging) of unstained cells of *Xanthomonas campestris* pv. vesicatoria strain 484 grown in the presence of 1.2 mM cupric sulfate. Top: ΔE = 0 eV; Bottom: ΔE = 930 eV.

operon of *Pseudomonas* shares homology to a region of copper-resistance genes from *X. campestris* pv. vesicatoria, however *Xanthomonas* genes were not expressed in *Pseudomonas*, indicating that they have diverged at the regulatory level (Mills *et al.*, 1993; Voloudakis *et al.*, 1993). The results we have obtained show that despite this divergence, a similar mechanism of copper accumulation also occurs in this strain of *X. campestris* pv. vesicatoria, as in *Pseudomonas*. It is likely that other mechanisms occur in *Xanthomonas*, since there are resistant strains showing no colony color alteration when grown in copper-containing medium.

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RESUMO

Foi analisada uma linhagem de *Xanthomonas campestris* pv. vesicatoria que apresentou resistência a sulfato de cobre 1.2 mM, através de espectrofotometria de absorção atômica e espectrofotometria eletrônica de imagem. Foi detectado acúmulo de cobre na periferia da membrana celular, sugerindo que o mecanismo de resistência ao cobre nesta linhagem é similar ao descrito para *Pseudomonas*. A análise da curva de crescimento também mostrou que o sistema é induzível, ocorrendo uma fase "lag" de cerca de 12 h.

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